University of Alberta

POLLEN CONTAMINATION, MATING SYSTEM, AND GENETIC DIVERSITY IN A *Pinus merkusii* SEEDLING SEED ORCHARD

By

Endah Suwarni ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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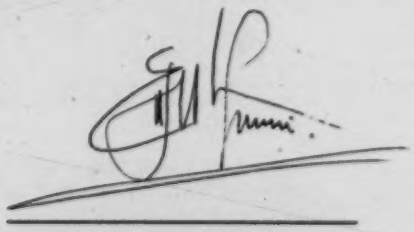

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I would like to dedicate this thesis to my mother Dra. Farial, my brother Nanang, and Najib, and my sister Dewi, as well as my special friend Kenneth Raiche, for their encouragement, love, prayer, and tremendous support to complete this work.

ABSTRACT

The genetic control of allozyme variants in *Pinus merkusii* Jungh. & De Vriese was studied. Using the established allozyme gene markers, pollen contamination, mating system, and genetic diversity parameters were estimated in a *P. merkusii* seedling seed orchard complex in East Java, Indonesia. A substantial level of contamination (29.7% ± 18%) from the surrounding stands was observed in the seed orchard. The outcrossing rates were very low, ranging from 0.14 to 0.31 and 0.10 to 0.24 for the multi-locus (t_m) and single-locus (t_s) estimates, respectively. The species showed relatively low genetic diversity. Gene diversity did not differ significantly between the seed orchard (He = 0.086 ± 0.002) and the surroundings stands (He = 0.088 ± 0.015).

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CHAPTER 1

GENERAL INTRODUCTION

Pinus merkusii

Pinus merkusii Jungh. & De Vriese is one of the tropical pines. Junghuhn discovered the species in the mountains of Sipirok, North Sumatra. He named it *Pinus sumatrana,* and Professor De Vriese published the first botanical description in 1845 and named the species *Pinus merkusii* Junghuhn & De Vriese (Cooling 1968; de Laubenfels 1988).

Geographic distribution and habitat

The natural distribution of *Pinus merkusii* extends from the mainland of Southeast Asia (Myanmar, Thailand, Vietnam, Laos, Cambodia) to Luzon and Mindanao Island in Philippines, and Sumatra Island in Indonesia (Fig. 1.1). It grows on a diversity of soils, generally on poor quality acid podzolic soils over sandstone or volcanic ash, sometimes on deeply leached acid basalt, rarely successfully competing on richer forest soils (Cooling 1968, de Laubenfels 1988).

This species has a large altitudinal range from a few meters above sea level to over 2,000 m, and under various climates from seasonal to constantly humid equatorial. Throughout the mainland range, and in the Philippine locations, the climatic conditions are markedly seasonal with a relatively high precipitation alternating with three to six months of dry season. Sumatra is an exception, since average monthly precipitation is above the rate of potential evapotranspiration throughout the year (Cooling 1968; de Laubenfels 1988).

Variation in *Pinus merkusii*

Although Mirov (1967) has described *P. merkusii* as a not very variable pine, Cooling (1968) found that phenotypic variation over its range of distribution point

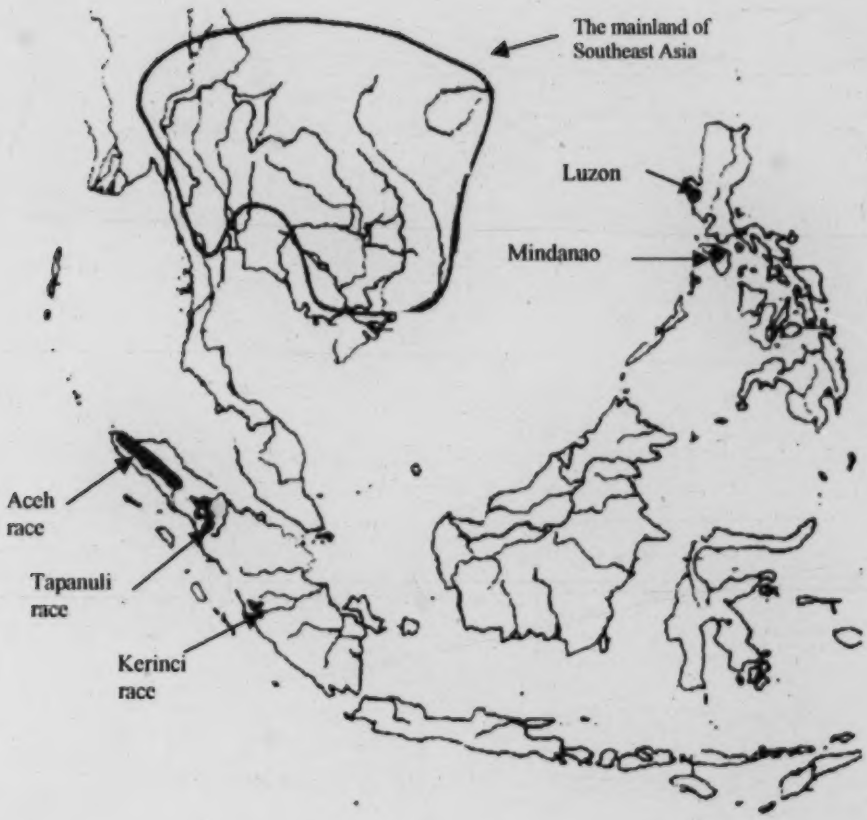

Fig.1.1 Range of natural distribution of *Pinus merkusii* Jungh & De Vriese, which covers the mainland of southeastern Asia, Luzon and Mindanao islands (Philippines) and Sumatra Island (Indonesia) (Cited from de Laubenfels 1988).

to the need for taxonomic review of this species. *P. merkusii* from the mainland Southeast Asia provenance, including the Philippines, showed markedly different phenotypes from those of the Sumatra provenance in respect to seed weight, presence or absence of a grass-stage in the seedlings, nodal habit, needle and cone dimensions, wood density patterns, oleoresin constituents, tree size and bole form, habitat and natural range (Table 1.1) (Cooling 1968).

The differences between the two provenances are of direct practical importance. There is also evidence of clinal, or ecotypic variation (Cooling 1968). This emphasizes the need for careful attention to provenance testing, and for source identification of all seed and forest products, including timber and oleoresin (Cooling 1968).

The populations of the Sumatra *P. merkusii* were divided into three races: *Aceh, Tapanuli*, and *Kerinci* (Fig.1.1). These are distinguished by geographical distribution but also by climatic conditions. The *P. merkusii* in Java originated from Sumatra, and almost exclusively from the *Aceh* race, and they were planted extensively using the taungya system. This agroforestry practice consists of forest plantations interplanted with food crops (Cooling 1968). Right now there are more than 584,000 ha *P. merkusii* plantation forests in Java (Perum Perhutani 1994).

A genetic inventory in 11 natural population of *P. merkusii* in Thailand revealed only little genetic diversity at 14 isozyme loci with an average *He* = 0.058. Allelic differentiation among populations was also small (δ=0.034), but higher than the differentiation reported from many conifers. Genotypic structures of the seed samples are characterized by a deficiency of heterozygotes relative to Hardy-Weinberg expectations in most populations. Estimation of outcrossing rates revealed an extraordinary high inbreeding ($0.017 < t_m < 0.65$ for nine out of ten populations analyzed). The low genetic diversity may be caused by a severe bottleneck in the evolutionary history of the populations (Changtragoon and Finkeldey, 1995).

Table 1.1. Summary of variation between two groups of provenances of *P. merkusii* Jungh. & De Vriese (de Laubenfels 1988)

Characteristics	Sumatra provenances	Continental provenances
Seedling	Slender stem, thin bark, little or no secondary cortex. Bark: xylem ratio 0.05.	Grass-like stage, very little stem elongation, but considerable thickening due to secondary cortex bark: xylem ratio 1.5-2.
Seed	Small, 17 g/1000	Medium-large, 24-35 g/1000
Growth habit	Multinodal (2-7 whorls/year)	Uninodal
Needles	Short, thin, light. 16-19 cm; 58-90 mg. per pair	Medium to long, sometimes heavy. 19-25 cm, 103-195 mg. per pair
Cones	Short to medium length (5.2-7.6 cm), light (3.2-11.7 g per cone). Cylindrical with bluntly pointed ends. Borne singly, in pairs, or in clusters of 3-7, occasionally up to about 20.	Medium to long (6. 7-12. 6 cm), medium weight to very heavy (14-48 g per cone). Elongate conic, greatest diameter at 1/3rd length. Borne singly, in pairs or some-times in three.
Bark	*Tapanuli* race: thin, smooth; *Aceh* race: thick and fissured	Usually very thick and fissured
Wood	Growth rings indistinct. Density variation early: late wood slight (0.7-0.11 density unit)	Growth ring conspicuous but very narrow. Density early: late considerable (0.3 - 0.4 unit)
Oleoresin	Simple chemical composition (α-pinene)	Complex chemical composition (α - pinene, Δ -3 carene, and others)
Tree size and bole form	55-60 m, bole often slender, spiral form in youth, straightening with age	Up to 40 m, straight cylindrical bole even in youth
Climate	Equatorial humid , about two months of dry seasons	Monsoon (humid to sub humid), six to months of dry seasons, with strong moisture deficit
Range	Sumatra islands only. Sea level to over 2,000 m above	Mainland southeast Asia includes Philippines. Sea level to 900 m

So far there is no information about genetic variation at the allozyme level in the natural populations of *P. merkusii* in Sumatra, which is the original source of this species. Therefore, it is necessary to study genetic variation existing in the natural populations of *P. merkusii* in Sumatra, and compare it with that existing in the production and breeding populations. This is to ensure that the *P. merkusii* seed orchard consists of broad genetic diversity, at least similar with that in the natural populations.

Products of *P. merkusii*

Exploitation of *P. merkusii* has been primarily for its resinous extractives and wood. The tree is known to produce a large quantity of oleoresin, as the resin canals are large and plentiful. The flow of resin may be quickened by application of sulfuric acid solution. Excessive tapping weakens the tree and reduces the value of the timber, but that is more than compensated for by a large production of resin, because the economic significance of the species lies in its good yield of high quality resin and turpentine. The wood is of high quality and generally is used for construction and decorative purposes, and also for pulpwood, flooring, and furniture (Cooling 1968, Andrew 1973).

OBJECTIVES OF THE RESEARCH

The main objectives of this study were to assess the level of pollen contamination and mating system parameters in a *P. merkusii* seed orchard (Chapter 3), and to determine the genetic diversity of the species (Chapter 4). Prior to estimating the pollen contamination and genetic diversity parameters by means of allozymes, it is important to analyze the genetic control of isozyme patterns, which is presented in Chapter 2. The different chapters are presented in the form of manuscripts, which have been or will be submitted to refereed journals for publication. Therefore, they are written according to the instructions of the target journal.

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CHAPTER 2

INHERITANCE OF ISOZYME VARIANTS IN SEED TISSUES OF *Pinus merkusii* Jungh. & De Vriese

INTRODUCTION

Pinus merkusii Jungh. & De Vriese is generally found throughout Asia from Myanmar through Thailand, Indochina, Sumatra and the Philippine Islands of Luzon and Mindanao (Mirov 1969; de Laubenfels 1988). In Java-Indonesia, there are more than 584,000 ha of *P. merkusii* plantation forests. It is one of the prime commercial species and has the highest priority in genetic tree improvement programs. Seedling seed orchards were established from plus trees of *Pinus merkusii* selected from all over Java. This species is fast growing, and its timber has been used widely for construction, flooring, carving and furniture. However, its major economic significance depends on its resin, since it is a notable source of resinous products and high quality turpentine (Andrew 1973).

The study of allozyme variation of *P. merkusii* from natural populations in Thailand and Vietnam revealed that this species had very low intrapopulational variability but high levels of interpopulational differentiation (Szmidt et al., 1996). It is necessary to establish reliable models of the inheritance of isozyme loci prior to studies of mating system and pollen contamination in *P. merkusii* seedling seed orchards. The megagametophyte tissue of conifer seeds is haploid, thus allowing direct observation of Mendelian segregation of allozymes of heterozygous trees (Guries and Ledig 1978, Adams and Joly 1980)

This paper describes the variation patterns of nine enzyme systems in *P. merkusii* and demonstrates the mode of inheritance at polymorphic allozyme loci. Included are tests for the inheritance of five polymorphic loci from each of 12 heterozygous mother trees, using segregation ratios.

MATERIALS AND METHODS

Plant material

Mature cones were collected from 100 *Pinus merkusii* trees from Sempolan - Jember- East Java, Indonesia. The seeds were extracted and kept separate by maternal parent and later shipped to the University of Alberta where they were refrigerated until laboratory analysis. The seeds were surface sterilized in 1 % (v/v) Javex for two minutes and rinsed with autoclaved water six times to prevent fungal growth. They were germinated on moistened filter paper at room temperature until the radicles had emerged 2-4 mm beyond the seed coat.

Electrophoretic procedure

The ideal sampling for examination of inheritance and linkage is to examine a large number of haploid megagametophytes from heterozygous trees. The megagametophytes and embryos from germinating seeds were excised from their seed coats, placed individually in 0.5 ml conical polystyrene sample cups (Elkay Products, Inc. 800 Boston Turnpike, Shrewsbury, MA). They were crushed with a knurled teflon grinding head in 40 µl extraction buffer. The extraction buffer consists of: 30 mM Tris, 5 mM citric acid, 0.4 mM β nicotinamide adenine dinucleotide (NAD), 0.2 mM β nicotinamide adenine dinucleotide phosphate-disodium salt (NADP), 1 mM ascorbic acid, 1 mM ethylene diamine tetraacetate disodium salt (EDTA), 0.1 % (w/v) bovine serum albumin (BSA), pH adjusted to 7 with 1 M citric acid (Khasa et al. 1993). The extract was absorbed onto 2x15 mm paper wicks (Beckman, model R) and inserted into a vertical slice of 12.5 % starch hydrolyzed gel (Pasteur Merieux Connaught, Rhone Poulenc Group).

Initially, ten megagametophytes and ten embryos from each of 100 trees were genotyped, and heterozygous trees were identified for each locus. An expected 0.195 % [$(½)^{10-1}$] of the heterozygotes would be miss-classified in this way, assuming 1:1 segregation at heterozygous loci. For this study, 12

heterozygous trees were selected and at least 45 haploid megagametophytes for each tree were analyzed.

Two electrophoretic buffer systems were used for the 12 enzyme systems assayed. These buffer systems (H and B) are described in Table 2.1. Aconitase, alcohol dehydrogenase, adenilate kinase, Isocitrate dehydrogenase, malate dehydrogenase, malic enzyme, 6-phosphogluconic dehydrogenase, phosphoglucomutase, and shikimate dehydrogenase were run with the H buffer system. Aspartate aminotransferase, colorimetric esterase, and leucine amino peptidase were assayed with the B buffer system. However, esterase and leucine aminopeptidase did not stain clearly. Considering consistency and cost effectiveness, only nine enzymes run with the H electrophoretic buffer system were retained for further analysis (Table 2.1). The details and references for staining recipes are described in Khasa (1993) and Liengsiri et al. (1990) with very important modifications, such as replacing NBT with MTT for SKDH, adding MTT for MDH and doubling the amount of ADP for AK enzyme (Appendix 1.)

Table 2.1 Buffer systems and electrophoretic conditions

Buffer System	Electrode buffer	Gel buffer	Electrophoretic conditions	Reference
H	0.125 M Tris pH adjusted to 7.1 with 1 M citric Acid	0.05 M Histidine HCl, 1.4 mM EDTA, pH adjusted to 7 with 1M Tris	I = 50 mA/gel T = 4-5 hours	Cheliak & Pitel (1984)
B	0.06 M Lithium hydroxide, 0.3M Boric Acid, pH adjusted to 8.5 with 1 N NaOH	0.03 M Tris 0.005M Citric Acid 1 % (v/v) electrode buffer B pH adjusted to 9 with 1 N NaOH	I = 30 mA/gel T = 4-5 hours	Cheliak & Pitel (1984)

Notes: H gel buffer is diluted in a 1:4 ratio before use
B gel buffer is diluted in a 1:9 ratio before use

Genetic analyses of isozyme and allozyme variants

The enzyme loci were identified by abbreviation and a hyphenated numeral. The relative mobility (Rf) of each locus was calculated as the relative migration distance of the common allozyme to the migration distance of the standard (hybrid corn span cross from Apache Seeds Ltd., Edmonton-Alberta). The most-anodally-migrating locus was designated as 1, the next most anodally migrating locus as 2, and so on. Within each locus, the most anodally-migrating allele was designated as 1, the second as 2, and so on. Any allozyme variant (Rfa) observed was measured relative to the common allozyme (Rf). The mean number of putative alleles per locus at the species level (As) was calculated following the formula $As = 1/m \Sigma a_i$, where m in the number of putative loci scored and a_i is the number of putative alleles observed at locus i.

The *G* statistic, which has an approximate Chi-square distribution with 1 df (Sokal and Rohlf, 1981) was used to detect deviation from the expected 1:1 segregation ratio, and G_H test to investigate heterogeneity among families. All tests of statistical significance were conducted at the $P \leq 0.05$ level.

RESULTS AND DISCUSSION

General comments

A total of 20 loci from nine enzyme systems run on the H buffer system were resolved consistently (Appendix 2) and scored for the 100 trees analyzed. Additional zones that appeared inconsistently in three enzyme systems run on the B buffer system were excluded. Five of twenty loci scored were polymorphic at 95 % criterion (*Idh-2, Mdh-2, Pgm-1, Pgm-3,* and *Skdh-1*; Table 2.2) in both megagametophyte and embryo tissues. The remaining loci were monomorphic (*Aco, Ak-2, Ak3, Mdh-1, Mdh-3, Me-1, 6-Pgd-2, Pgm-2, Skdh-2, Skdh-3*), or nearly monomorphic with rare alleles those were found in a heterozygous state for *Adh-3, Ak-1, 6-Pgd-1, Me-2,* and *Idh-1.*

Table 2.2 Enzyme systems for which electrophoretic bands were resolved consistently and allozyme variants of *P. merkusii*

Enzyme	EC Number **	Locus	Alleles 1	2	3	4
Aconitase (ACO)	4.2.13	*Aco*	100			
Alcohol dehydrogenase (ADH)	1.1.1.1	*Adh-3*	106*	100		
Adenilate kinase(AK)	2.7.4.3	*Ak-1*	157	129*	114*	100*
		Ak-2	100			
		Ak-3	100			
Isocitrate dehydrogenase (IDH)	1.1.1.42	*Idh-1*	110*	100		
		Idh-2	132	100	80	
Malate dehydrogenase (MDH)	1.1.1.37	*Mdh-1*	100			
		Mdh-2	116	100		
		Mdh-3	100			
Malic enzyme (ME)	1.1.1.40	*Me-1*	100			
		Me-2	110*	100		
6-Phosphogluconic	1.1.1.44	*6-Pgd-1*	108*	100		
dehydrogenase (6-PGD)		*6-Pgd-2*	100			
Phosphoglucomutase (PGM)	2.7.5.1	*Pgm-1*	100	87	81	
		Pgm-2	100			
		Pgm-3	126	100		
Shikimic acid dehydrogenase	1.1.1.25	*Skdh-1*	106	100	91	
(SKDH)		*Skdh-2*	100			
		Skdh-3	100			

* Rare alleles

** The enzyme commission numbers (E.C) follow the International Union of Biochemistry (1984)

We found more polymorphic loci than reported by Changtragoon and Finkeldey (1995) who found only two polymorphic loci out of 13 loci, and the remaining loci were dominated by the same frequent allele in all populations with one or two rare alleles in some populations.

The number of band phenotypes observed in embryo tissues varied from two to four bands, and megagametophyte band phenotypes at a locus varied from single to double banded. The number of alleles observed at polymorphic loci varied from two to three (Table 2.2). Coelectrophoresis of embryos and megagametophytes showed that the same genes were expressed in both tissues, except for *Skdh-3* and *Mdh-1* where I observed some variability between the two tissues. The mean number of putative alleles per locus at the species level (As) was 1.7, and *Ak-1* had the highest number of alleles per locus.

Description of enzyme systems

Aconitase (ACO)

ACO was observed as a monomeric enzyme with one monomorphic locus (Rf = 0.78, Fig. 2.1). This result agrees with the study by Schmidt et al. (1996) in natural populations of *Pinus merkusii* from Thailand and Vietnam. Two zones of activity have been detected in other conifers (El Kassaby et al. 1982). One zone of activity was found in Whitebark pine (*Pinus albicaulis* Engelm.) with three alleles (Furnier et al. 1986).

Alcohol dehydrogenase (ADH)

As observed in other conifers (see among others Xie et al. 1991), three loci were obtained for this enzyme (Rf = 3.66, 3.1, and 2.5) but only one locus, *Adh-3,* can be scored (Fig. 2.1). *Adh-1* and *Adh-2* were very faint and occasionally did not stain, and therefore were eliminated from this study. Heterozygous embryos had three bands, while the megagametophyte phenotype had a single band, indicating that ADH is a dimeric enzyme. In whitebark pine, Furnier et al. (1986) found only one zone of activity for ADH, with two segregating alleles.

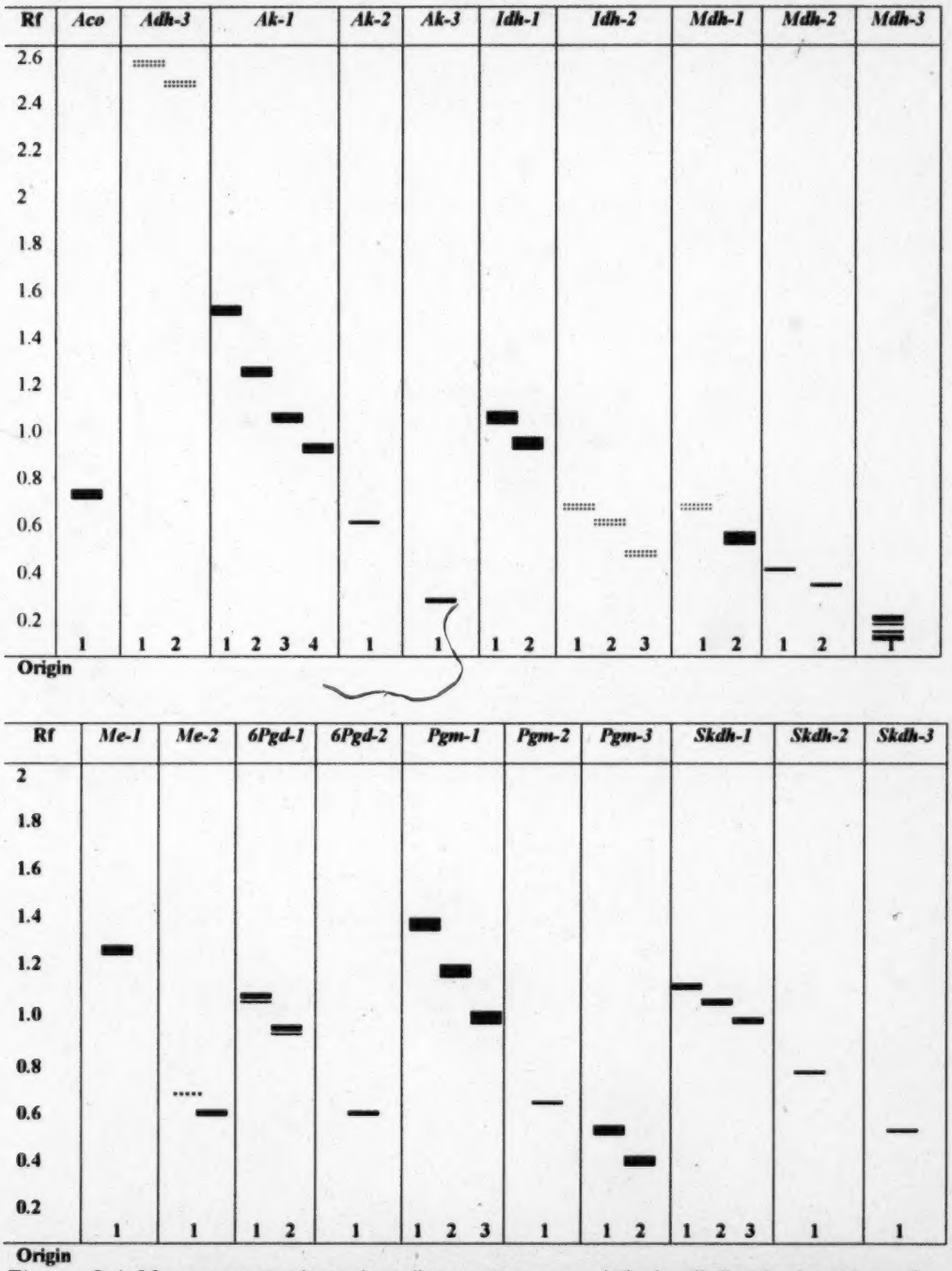

Figure 2.1 Megagametophyte banding patterns and their allelic designations for 20 loci in *P. merkusii*. Rf is the migration distance relative to the standard (hybrid corn); dashed lines indicate faint bands.

Adenilate kinase (AK)

Three zones of activity were found for AK (Rf = 0.9, 0.66, and 0.36, Fig. 2.1). The phenotype of the heterozygous embryo was double banded in *Ak-1*, while the megagametophyte phenotype was single-banded. The quaternary structure was inferred to be a monomeric enzyme. Three rare alleles were observed in heterozygous embryos in *Ak-1*; allele 3 and 4 occurs at less than 1 % and allele 2 occurs at 2,1 %. *Ak-2* and *Ak-3* were monomorphic both in megagametophyte and embryo tissues.

Isocitrate dehydrogenase (IDH)

It has been reported that IDH has only a single-locus in conifers and rarely is polymorphic (El Kassaby et al., 1982), except in coastal Douglas-fir (*Pseudotsuga menziesii* var. menziesii (Mirb) Franco) (Yeh and O'Malley 1980) and White spruce (*Picea glauca* (Moench) Voss) (King and Dancik 1983), where IDH is highly polymorphic. In this study, IDH was observed to have two loci (Rf = 1.04 and 0.66, Fig. 2.1), consistent with the report by Xie et al. (1991) in *Thuja orientalis* Linn. The first locus is monomorphic and strongly stained. The second has three alleles and is a bit weakly stained. Heterozygous embryos showed triple-banded isozyme patterns, while the megagametophyte phenotype had one strongly staining band, plus a weakly staining trailer band, suggesting that IDH is functionally dimeric. Similar observations were also made by Millar (1985) in Bishop pine (*Pinus muricata* D. Don).

Malic enzyme (ME)

Malic enzyme has two loci and both of them were invariant (Rf = 1.30 and 0.70, Fig. 2.1). The second locus (*Me-2*) was stained more intensely than the first, but it was inconsistent. El-Kassaby et al. (1982) also observed two zones of activity in coastal Douglas-fir (*Pseudotsuga menziesii var. menziesii*) for ME. In *Thuja orientalis* L, however, one zone of activity with three single-banded allozyme variants was observed (Xie et al. 1991). Keppart (1990) reported that this enzyme is tetrameric in many plants.

Malate dehydrogenase (MDH)

Four zones of activity appeared in MDH. However, isozyme activity was explained assuming three loci for MDH (Rf = 0.62, 0.38, and 0.23, Fig. 2.1.). The most anodal band (*Mdh-1*) was intensely staining. In addition to the common allele, a fast variant was observed in embryos, but absent in megagametophyte tissues indicating some variability of gene expression between the two tissues or that the locus is expressed in the megagametophyte but not detected by electrophoresis. The second zone indicated a locus (*Mdh-2*). Embryo alleles stained at different mobility (faster) than the megagametophyte alleles in this locus. This means that the genes were differentially expressed or modified in the two tissues. The two slowest zones appeared to represent a single-locus (*Mdh-3*) and a heterodimer between *Mdh-2* and *Mdh-3*. The same result was observed on the study on white spruce (King and Dancik 1983) and Douglas-fir (El-Kassaby et al. 1982). *Mdh-1* and *Mdh-3* were invariant, and the second locus (*Mdh-2)* had 2 alleles.

6-Phosphogluconic dehydrogenase (6-PGD)

There were two zones of activity in 6-PGD (Rf = 1.00 and 0.65, Fig. 2.1.). The first locus showed triple bands in heterozygous embryos and one banded phenotype with a shadow-band in the megagametophyte, suggesting that 6-PGD is dimeric. In other conifers 6-PGD was also described as dimeric (Xie et al. 1991). Neale and Adams (1981), Adams and Joly (1980), and Furnier et al. (1986) found only one monomorphic locus for 6-PGD in loblolly pine (*Pinus taeda*. L), balsam fir (*Abies balsamea*) and whitebark pine. However, King and Dancik (1982) observed two polymorphic loci in 6-PGD in white spruce (*Picea glauca*).

Phosphoglucomutase (PGM)

There were three PGM loci observed (Rf = 1.4, 0.77, and 0.50, Fig. 2.1.). *Pgm-1* and *Pgm-3* had three and two alleles, respectively. *Pgm-2* was monomorphic. The heterozygous embryos were strongly double-banded, while

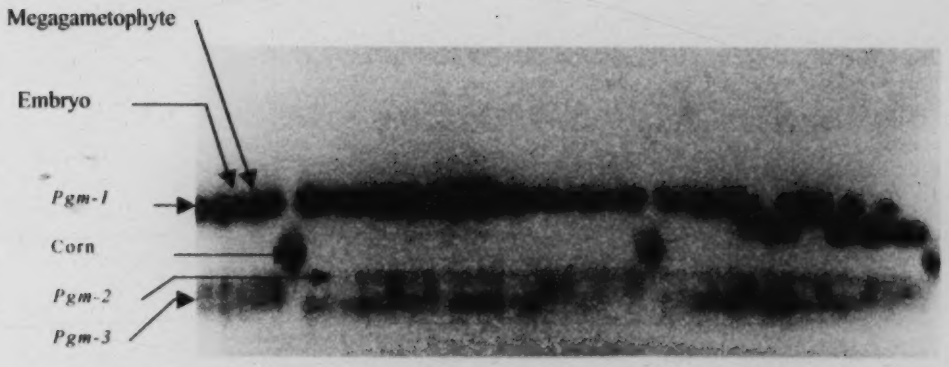

Figure 2.2 PGM enzyme showing three zones of activity. *Pgm-1* and *Pgm-3* are polymorphic, *Pgm-2* is invariant.

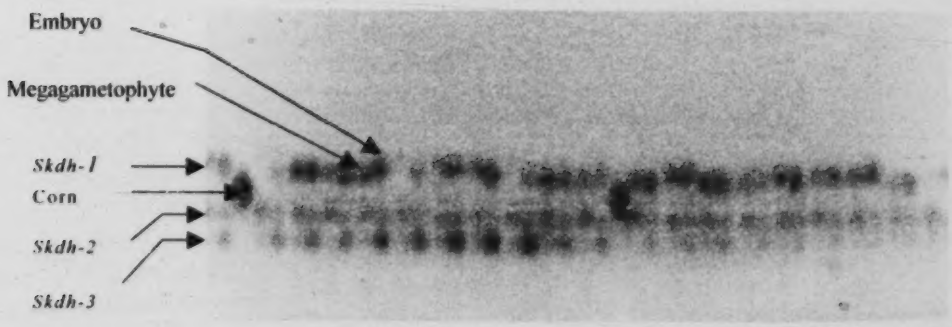

Figure 2.3 SKDH enzyme displayed three zones of activity. *Skdh-1* is polymorphic, while *Skdh-2* and *Skdh-3* are invariant. The embryo tissue at *Skdh-3* showed a strong banding pattern, but the megagametophyte was slightly stained.

megagametophyte phenotype was single-banded, indicating that the quaternary structure of this enzyme is monomeric. Only one highly polymorphic zone of activity for PGM appeared in white spruce (King and Dancik 1983). Two loci encoding PGM have been reported for many other conifers (Guries and Ledig 1978; Neale and Adams 1981; Xie et al. 1991, and Szmidt et al. 1996). *Pgm-1* was observed to be invariant, and *Pgm-2* had two alleles in *Pinus merkusii* as reported by Szmidt et al. (1996). This is in contrast to our findings, where we found three clear putative loci (Fig. 2.2)

Shikimic acid dehydrogenase (SKDH)

Three zones of activity were detected in SKDH (Rf = 1.13, 0.83, 0.63; Fig. 2. 1). *Skdh-1* had three alleles, and heterozygous embryos formed two-banded patterns. The structure is typically monomeric. *Skdh-2* and *Skdh-3* were invariant and this also occurred in Bishop pine (Millar 1985). Isozyme activity showed substantial variability between megagametophyte and embryo tissues in *Skdh-3*. The embryo tissue displayed a strongly stained banding pattern, while the megagametophyte tissue was slightly stained (Fig. 2.3). Szmidt et al (1996) scored consistently only one locus, *Skdh-1*, whereas *Skdh-2* showed poor reproducibility.

Segregation analyses

Observed single-locus segregation of allozymes from megagametophytes of heterozygous mother trees of *P. merkusii* and *G-tests* for goodness of fit to the 1:1 ratio and heterogeneity among families (G_H) are given in Table 2.3.

On the basis of the *G-tests*, there was only one locus (*Pgm-1*) that had a significant deviation from the expected 1:1 segregation ratio of allozymes (100/81 allelic combination). Such deviation can be caused by a number of factors, including meiotic drive, selection between meiosis, the time at which the seeds were assayed, linkage to a deleterious allele at another locus or experimental error (Furnier at al. 1986; Strauss and Conkle 1986; Xie et al. 1991). The

remaining polymorphic loci indicated simple Mendelian segregation in their mode of inheritance.

Table 2.3 Observed single-locus segregation of allozymes from megagametophytes of heterozygous mother trees of *P. merkusii* and *G-tests* for goodness of fit to the 1:1 ratio and heterogeneity among families G_H.

Locus	Allelic Combination	Observed segregation	Deviation		Heterogeneity	
			$G(1)$	$(P=\alpha)$	G_H(df)	$(P=\alpha)$
Idh-2	132/100	86/74	0.9	>.1	0.422(2)	>.1
	100/80	25/39	3.06	>.05	-	
	132/80	104/98	3.18	>.1	2.824(2)	>.1
Mdh-2	116/100	118/147	3.17	>.05	3.558(3)	>.1
Pgm-1	100/87	170/149	1. 38	>.1	5.747(4)	>.1
	100/81	64/43	4.15	<.05	10.498(1)	<.001
Pgm-3	126/100	347/392	2.74	>.1	6.59(11)	>.1
Skdh-1	106/100	58/54	0.14	>.1	0.504(2)	>.1
	100/91	61/84	3.66	>.05	0.564(1)	>.1
	106/91	54/66	1.2	>.1	0.312(1)	>.1

CONCLUSIONS

The twenty-isozyme loci described for *Pinus merkusii* comprised five polymorphic loci, with the remaining loci monomorphic. In a previous study, Szmidt et al. (1996) found only nine polymorphic loci (0.95 criterion) out of 16 loci in at least one population examined. Most loci showed low polymorphism, which is in agreement with our findings.

Most of the allozyme variants were expressed in both megagametophyte and embryo tissues, except for *Skdh-3, Mdh-1,* and *Mdh-2*. From the *G-tests* performed on polymorphic loci, there was one pairwise allelic combination, *Pgm-1* (100/81), which showed deviation from a 1:1 segregation ratio. The

confirmation of a 1:1 segregation ratio in heterozygous families supports the contention that these allozymes exhibit Mendelian inheritance in a codominant fashion. These loci stained clearly and consistently and can be used for many purposes in population genetics studies including mating system and pollen contamination assessments in seed orchards.

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CHAPTER 3

ESTIMATION OF POLLEN CONTAMINATION AND MATING SYSTEM IN A *Pinus merkusii* Jungh. & De Vriese SEEDLING SEED ORCHARD BY ALLOZYME MARKERS

INTRODUCTION

Pinus merkusii Jungh. & De Vriese is one of the tropical pines in Southeast Asia. Its natural distribution ranges from the mainland of Southeast Asia, which includes Myanmar, Thailand, Vietnam, Laos, Cambodia, all the way down to Luzon and Mindanao Island in Philippines, and Sumatra Island in Indonesia. It is found on a diversity of soils, over a large altitudinal range and under various climates (Cooling 1968; de Laubenfels 1988; Mirov 1969).

The Sumatran populations are distinguished into three races: *Aceh, Tapanuli*, and *Kerinci*. The *Aceh* race had been extensively planted in Sumatra and Java using the agroforestry taungya system (Cooling 1968). Right now there are more than 584,000 ha of *P. merkusii* plantation forests all over Java, and this is one of the species that has the highest priority in genetic tree improvement programs for traits such as growth rate, stem straightness, and resin production.

A seed orchard is a plantation of selected clones or progenies, which is isolated or managed to avoid or reduce pollination from outside sources, and is designed to produce frequent, abundant and easily harvested genetically improved seed for reforestation (Fielberg and Soegaard 1975).

To ensure maximum benefits from a seed orchard, both in seed production and seed quality, several assumptions must be met (Eriksson et al. 1973; El-Kassaby et al. 1983; Blush et al. 1993). These include: synchronization of flowering, isolation from surrounding stands, equality of male and female gamete production, random mating, equal compatibility for all crosses, and minimal self-fertilization.

The common problems in seed orchard management associated with pollination are inbreeding risk, amount and composition of cross-pollination, and pollination from sources outside the orchard (Koski 1975). Therefore seed orchards should be designed as much as possible to permit maximum cross-pollination among members of the orchard and be protected from background pollen, which is mainly derived from surrounding stands of the same species (Koski 1975; Zobel and Talbert 1991).

Pollen contamination is probably the most important factor that reduces genetic efficiency of a seed orchard (Friedman and Adams 1982). Most studies of wind-pollinated orchards indicate that between 25-80 % of seeds produced resulted from fertilization by contaminant pollen (Lowe and Wheeler 1993). In young orchards in which natural pollen production is minimal, the contamination rate could be even higher. Pollen contamination may reduce the seed orchard genetic gains that are expected under complete panmixia (Fast et al. 1986). The estimates of genetic loss are based on the assumption that the contaminating pollen is unimproved and poorly adapted to the region where the seed will be used (Blush et al. 1993; Lowe and Wheeler 1993).

Several techniques have been used for reducing pollen contamination, such as supplemental mass pollination (SMP), bloom delay, flower stimulation, neighborhoods, pollen mixing, selective harvesting of cones, amelioration and controlled pollination (Lowe and Wheeler 1993; Wheeler et al. 1993). However, the critical factor is isolation from genetically unimproved pollen from outside sources. Isolation can be accomplished phenologically or geographically. Whenever it is possible, orchards should be established outside the species range, in areas where the species is scarce, or in areas where phenological overlap of the orchard and surrounding populations is nil. Otherwise, the isolation strips around the orchards should be as large as possible (Koski 1987; Lowe and Wheeler 1993).

The genetic efficiency of a seed orchard also depends on the mating success of individual orchard trees, or patterns of genetic relatedness between male gametes and female gametes that form the zygotes. The mating system

can change the genic and genotypic pools of an individual plant progeny and an overall population. Random mating (panmixia) results in genetic equilibrium as determined by the familiar random proportionality rules (Spiess 1989). The conditions set for panmixia include that individuals must mate without bias based on phenotypic resemblance, relatedness, or physical distance between them. Under non-random conditions, genic elements (i.e. alleles, gametic combinations, chromosomal variants and the like) will not form zygotes independently by simple proportional rules (Spiess 1989).

As reviewed by Khasa et al. (1993), several authors have reported detrimental effects of departures from random mating when using open-pollinated progenies in planting trials. First, inbreeding depression for economically and adaptively important characteristics may result in decreased height growth and vigor, poor survival, susceptibility to pests, reduced seed set, and high frequency of various dwarfs, spots, and pigment abnormalities. Second, estimates of variance components become biased when inbreeding occurs, resulting in erroneous estimations of heritabilities and genetic gains because of greater degree of relatedness among individuals within progenies than of the half-sib.

Indirect (e.g. pollen traps) and direct (e.g. genetic markers) methods have been used to measure mating system parameters and to estimate pollen contamination in a seed orchard (Ritland and El-Kassaby 1985). Allozyme markers and monoterpenes have been used as direct methods to assess pollen contamination in several seed orchards (Lindgren 1991). Each of these studies used a different analysis method suited for a specific situation. For example, Friedman and Adams (1981) identified allozymes unique to the surrounding natural trees (contamination), and to orchard trees other than the seed parents. Squillace and Long (1981) identified recombinant genotypes in orchard progenies which could not have been produced from legitimate orchard crosses. Because of the rarity of unique markers, the applicability of these early estimation techniques is limited. Identification of pollen parents by genetic exclusion has

also been used, but complete genetic exclusion is rarely possible, and without complete exclusion, paternity estimates are expected to be biased (Brown 1990).

El-Kassaby and Ritland (1986) introduced a method to estimate pollen contamination, which incorporates information provided by allelic differences unique to each pollen source (orchard and non orchard) and also quantitative allelic differences. Stewart (1994) presented a probability model technique based on multilocus gamete frequency differences among pollen sources that simultaneously estimates pollen contamination, pollen fertilities, and selfing rates of orchard trees.

Therefore, the purpose of this study is to estimate the level of pollen contamination and mating system parameters in a *P. merkusii* seedling seed orchard based upon differences between single-locus outcrossing pollen (male) vs ovule (female) gene frequency estimates (El-Kassaby and Ritland 1986). Based on these findings, a discussion or the use of the seed and management strategies could be proposed.

MATERIALS AND METHODS

Seed orchard description

The open-pollinated seedling seed orchard is located in Forest District Jember, Sub Forest District Sempolan, East Java-Indonesia, and is owned by Perum Perhutani (State Forest Company).

The orchard was established from 1978-1984 to improve traits such as growth rate, stem straightness, and resin production. It was designed for a half-sib progeny test and was initially composed of 1,000 families that were derived from selected plus trees of *Pinus merkusii* from all over Java. The total area covered approximately 96 ha, and was divided into six sectors (I-VI, Fig. 3.1) based on the year of planting. Each sector was 16 ha in size, which was split into 10 blocks (1-10, Fig. 3.1), with 1.6 ha per each block. Starting in 1978, 200 families that consisted of 5 seedlings per family were planted in each block of sector I. They were arranged in a randomized block design with blocks as

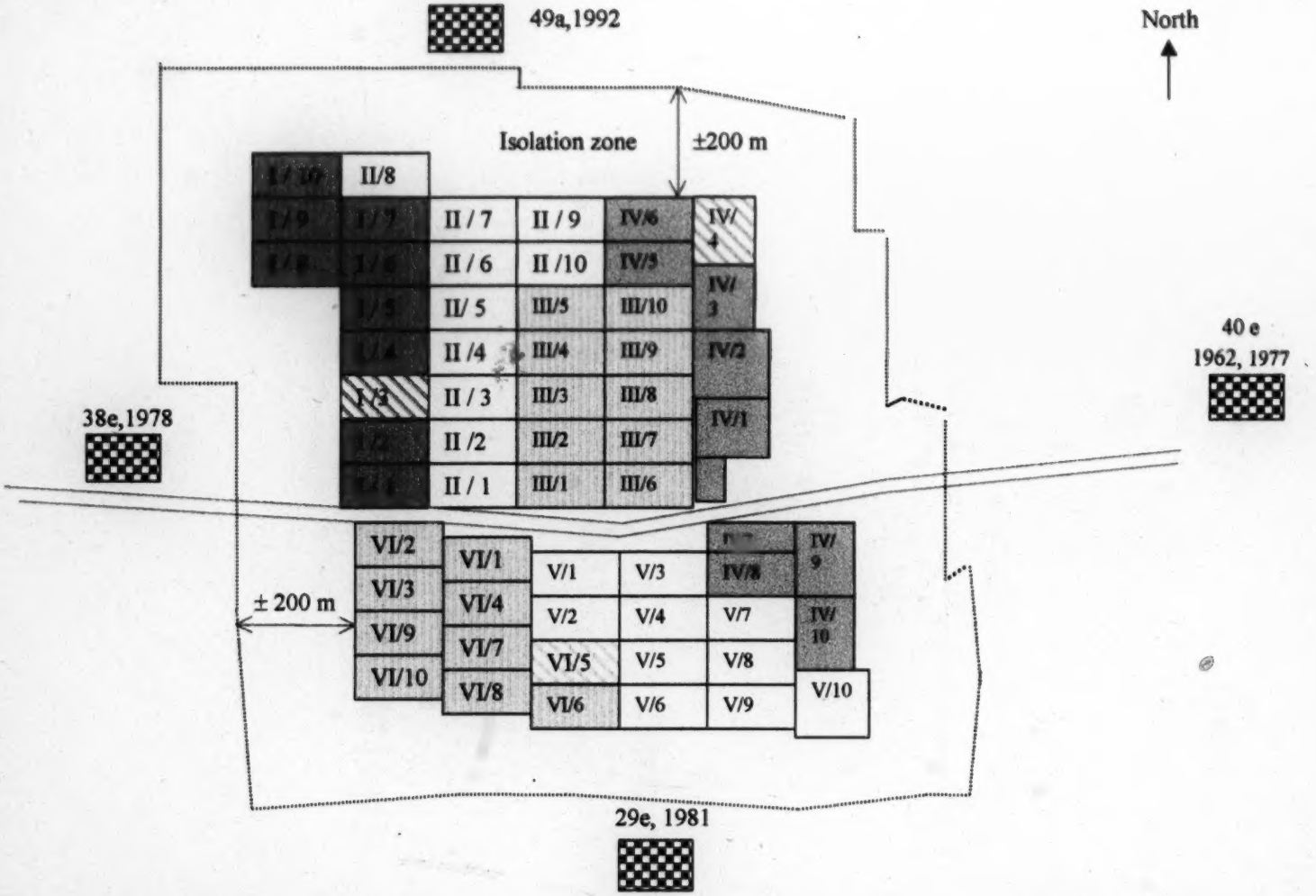

Figure 3.1. Map of the open-pollinated seedling seed orchard of *Pinus merkusii* in Jember Forest District, East Java, Indonesia

Legend of the map of the open-pollinated *Pinus merkusii* seedling seed orchard complex in Forest District Jember, East Java, Indonesia.

I - VI = Sectors referring to plantation established in 1978,1979, 1980, 1981, 1982, and 1983, respectively.

1 - 10 = Blocks (replications)

= The sample location in the local stands

= The sample block established in 1978

= The sample block established in 1981

= The sample block established in 1983

= Isolation zone boundary

= Main road

replications. In 1979, 40 families that were already tested in the previous year were planted in sector II, along with 160 new selected families. The same design was applied for sector III, IV, V and VI (Suseno 1984). The initial spacing was 4 x 4 m but at present the distance between trees is irregular because of roguing (Appendix 4).

Roguing has been done several times, and at the time the samples were collected, every block had a different number of families, with one or two trees per family. However there is no accurate record of the total number of families and trees left after roguing in this orchard. The final objective of the management plan is to have 50 families with 1 tree per family in each block.

The orchard was surrounded by a 200 m wide strip isolation zone, which consisted of a plantation of different species of different ages. These trees include: *Racosperma mangium* (1986 and 1983), *Calliandra callothyrsus* (1984), *Eucalyptus deglupta* (1986-1987), *Eucalyptus urophylla* (1981-1985), *Leucaena leucocepala* (1985), *Santalum album* (1986), *Swietenia mahagoni* (1981), *Tectona grandis* (1988), *Pinus merkusii* (1988), and mixed forest of the species mentioned above (1985) (Suseno 1984).

Beyond the isolation zone there were unselected forest stands of different forest tree species of various ages, but mainly *P. merkusii* that were planted in 2 x 3 m planting distance for wood and resin production.

Seed collection

Cones were collected from both genetically selected *P. merkusii* trees in the seed orchard and from unselected *P. merkusii* local stands surrounding the orchard, which are a putative source of pollen contamination (Fig. 3.1). The sample collection was carried out in August and September 1997.

From inside the orchard, cones were obtained from trees in three different blocks that were randomly sampled from different years of plantation as shown in Fig. 3.1. They were from sector I plantation (1978) block 3 (I/3); sector IV plantation (1981) block 4 (IV/4); and sector VI plantation (1983) block 5 (VI/5). Forty trees that represent 40 families were sampled per block.

From the area surrounding the orchard, 20 pine trees were sampled from forest stands No.38e, No. 40e, No. 49a, and No. 29e, which were located west, east, north, and south of the orchard, respectively. The samples from forest stands No. 38e, 40e and 29e were located almost immediately adjacent to the isolation zone (about 200 m), and forest stand No 49a was located about 400 m from the seed orchard (Fig.3.1). These background stands were pine plantations established in 1979, 1962, 1992, and 1981 for the west, east, north, and south, respectively (Appendix 5).

The cones were air-dried in sunlight, separated by family; then the seeds were extracted, cleaned by hand, and stored at 4° C until electrophoretic analysis at the University of Alberta.

Electrophoretic procedure and data analyses

Ten seeds per tree were electrophoretically assayed. The probability of incorrectly identifying a heterozygote at a particular locus is $(1/2)^{k-1}$, for k megagametopytes assayed per tree. With 10 seeds assayed per tree, the probability of miss-classifying a heterozygote is 0.195 %. Both megagametophyte and embryo tissues were run side-by-side in gel electrophoresis. In this way the genotype contribution from the male and the female parents to the seed orchard can be identified. The electrophoretic procedures, banding pattern of the allozymes, and the mode of inheritance were described previously (Suwarni et al 1999).

To estimate the level of pollen contamination, the genotype of each sample tree was inferred from its progeny array for five isozyme loci encoding four enzyme systems. The four enzymes were isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC. 1.1.1.37), phosphoglucomutase (PGM, EC. 2.7.5.1), and shikimate dehydrogenase (SKDH, EC. 1.1.1.25). To estimate mating system parameters, six isozyme loci from five enzyme systems were analyzed, which include the previous five enzymes plus adenilate kinase enzyme (AK, EC.2.7.4.3).

The allele frequencies of the five allozyme loci (*Adh-2, Pgm-1, Pgm-3, Mdh-2 and Skdh-1*) for the maternal parents (ovule pool), the outcrossing pollen parents (the pollen pool), and allele frequencies of the background stands were calculated using the Multilocus Mating System (MLTR version 1.0) computer program of Ritland (1998) (http://forgen.forestry.ubc.ca./ritland/programs.html). The pollen contamination rate was estimated by using the migration model by El-Kassaby and Ritland (1986). A Chi square contingency test (Ott 1993) was used to determine the heterogeneity of gene pools at the $p \leq 0.05$ significance level.

According to the migration model, the frequencies of the most common allele in the maternal tree population, together with the frequencies of the orchard (blocks) outcrossing pollen gene pools and the outside source (natural stands), were used to compute the contamination rate at locus *i*,

$$\hat{m}_i = \frac{p_i - r_i}{q_i - r_i}$$

where m_i is the estimated migration rate of pollen at the i^{th} locus or the proportion of natural stand pollen present in the orchard, r_i, is the frequency of the most common allele in the orchard ovule pool at the i^{th} locus, p_i is the frequency of the same allele in the orchard outcrossing pollen pool at the i^{th} locus, and q_i is the frequency of the same allele in the outside source at the i^{th} locus. Since p, r and q are independent samples of the genes, the variance of m_i is computed using the differential approximation without covariance term as follows:

$$v(\hat{m}_i) = \left[\frac{p_i(1-p_i)}{N_p}\right]\left[\frac{1}{q_i - r_i}\right]^2 + \left[\frac{r_i(1-r_i)}{N_r}\right]\left[\frac{(q_i - p_i)}{(q_i - r_i)^2}\right]^2 + \left[\frac{q_i(1-q_i)}{N_q}\right]\left[\frac{p_i - r_i}{(q_i - r_i)^2}\right]^2$$

where N_p is number of pollen (number of embryos), N_r is the number of ovules (number of trees x 2), and N_q is the number of foreign pollen (number of trees in the local stands x 2)

Assuming that the migration estimates are approximately independent among loci, the minimum variance estimate of migration on average over loci is the weighted average:

$$\hat{\bar{m}} = \left[\sum_{i=1}^{n} \frac{1}{v_{\hat{m}_i}}\right]^{-1}\left[\sum_{i=1}^{n} \frac{\hat{m}_i}{v_{\hat{m}_i}}\right]$$

where n is the number of loci studied. The variance of the minimum variance estimate is:

$$v(\hat{\bar{m}}) = \left[\sum_{i=1}^{n} \frac{1}{v_{\hat{m}_i}}\right]^{-1}$$

To estimate the mating system in each local stand and each block in the seed orchard, the MLTR version 1.0 computer program of Ritland (1998) was also used to obtain the multilocus outcrossing rate (t_m), and single-locus outcrossing rate (t_s) based on the six polymorphic loci (*Ak-1, Idh-2, Pgm-1, Pgm3, Mdh-2 and Skdh-1*). Standard errors of the outcrossing estimates were calculated using 200 bootstraps (re-sampling between families). The same set of polymorphic loci was used to estimate Wright's fixation index (Wright 1951), observed heterozygosity (*Ho*) and expected heterozygosity (*He*) in each block and local stand by running the data with POPGENE version 1.21 computer program of Yeh et al. (1997). Wright's fixation index, which is calculated as

$$F = 1 - \frac{Ho}{He}$$

measures the deviation of observed heterozygotes from the proportion expected under Hardy-Weinberg equilibrium. The proportion of the expected heterozygotes was calculated following the formula:

$$He = \frac{2N}{2N - 1}\left(1 - \sum_{i=1}^{n} p_i^2\right)$$

where N is the number of embryos sampled from the population and p_i is the frequency of the i^{th} allele at the locus (Nei and Roychoudhury 1974). The Chi-square (χ^2) test (Ott 1993) was employed to test the excess or deficiency of observed heterozygotes relative to Hardy-Weinberg proportions for statistical significance. The test was done at $p \leq 0.05$ significance level.

RESULTS

The estimates of allele frequencies for maternal parents or ovule pool (r), the outcrossing pollen parents (p) in the seed orchard, and background stands (q) are presented in Table 3.1, 3.2, and 3.3, respectively.

Table 3.1 The estimation of the out-crossing pollen pool allelic frequencies (p) among the three blocks in the seed orchard

Locus	Allele	Sector/Block			Chi-sq *
		I/3	IV/4	VI/5	
Idh-2	1	0.810	0.869	0.876	20.38**
	2	0.043	0.025	0.000	
	3	0.147	0.106	0.124	
Mdh-2	1	0.954	0.866	0.962	130.89**
	2	0.046	0.134	0.038	
Pgm-1	1	0.672	0.928	0.895	
	2	0.328	0.049	0.105	145.52**
	3	0.000	0.023	0.000	
Pgm-3	1	0.536	0.647	0.516	16.22**
	2	0.464	0.353	0.484	
Skdh-1	1	0.510	0.539	0.540	12.55**
	2	0.174	0.155	0.228	
	3	0.316	0.306	0.232	
Np		400	400	400	

* Chi square df = (r-1)(c-1), ** Denotes significant at 0.01 level

The chi-square contingency test was conducted to test the variability of gene pools among each of the three blocks inside the seed orchard and among the four stands surrounding the orchard. The outcrossing pollen and ovule pools

Table 3.2 The estimation of the ovule pool allelic frequencies (r) among the three blocks in the seed orchard

Locus	Allele	Sector/Block I/3	IV/4	VI/5	Chi-sq.
Idh-2	1	0.788	0.837	0.837	1.2*
	2	0.050	0.025	0.038	
	3	0.162	0.138	0.125	
Mdh-2	1	0.837	0.825	0.825	0.06
	2	0.162	0.175	0.175	
Pgm-1	1	0.800	0.837	0.887	5.95**
	2	0.175	0.112	0.112	
	3	0.025	0.050	0.000	
Pgm-3	1	0.650	0.538	0.512	3.51*
	2	0.350	0.463	0.488	
Skdh-1	1	0.825	0.563	0.512	27.79**
	2	0.125	0.325	0.225	
	3	0.050	0.112	0.262	
Nr		80	80	80	

* Denotes significant at 0.05 level; ** Denotes significant at 0.01 level

Table 3.3 The estimation of allele frequencies among the four *P. merkusii* backgrounds stands

Locus	Allele	Background stand East	South	West	North	Chi-sq
Idh-2	1	0.955	0.591	0.982	1.000	43.97**
	2	0.000	0.036	0.00	0.000	
	3	0.045	0.373	0.018	0.000	
Mdh-2	1	0.913	0.613	0.900	0.938	20.96**
	2	0.087	0.387	0.100	0.062	
Pgm-1	1	0.913	0.627	0.882	0.883	14.92**
	2	0.087	0.373	0.118	0.117	
	3	0.000	0.000	0.000	0.000	
Pgm-3	1	0.504	0.700	0.752	0.519	8.05**
	2	0.496	0.300	0.248	0.481	
Skdh-1	1	0.577	0.542	0.804	0.832	14.46**
	2	0.304	0.262	0.157	0.098	
	3	0.120	0.197	0.040	0.070	
Nq		40	40	40	40	

** Denotes significant at 0.01 level

were significantly heterogeneous among the seed orchard blocks (Table 3.2). The four background stands were also found to be heterogeneous (Table 3.3); therefore, each local stand can be considered as a source of background pollen separately that contributes to the contamination rate in each block of the seed orchard.

The estimated level of pollen contamination from adjacent unselected stands in the seed orchard for block-local stand combinations ranged from -10.3 % to 33.5 % (Table 3.4). The rate of the contamination averaged over blocks varied from - 21.7 % to 30.8%, with significant contribution coming from the east and the north local stands, and little contamination from the west and the south side. Under the model assumptions, negative values can only be caused by statistical sampling error (El-Kassaby and Ritland 1986). The contamination rates averaged over stands varied from 22.0 % in block VI/5 to 50.5 % in block IV/4. The overall estimate of the pollen contamination rate in this orchard was 29.7 % ± 18 %, and all of the background pollen sources contributed to this level of contamination with significant contribution from east and north local stands.

Table 3.4 Estimation of the pollen contamination rates and their 95% confidence intervals.

Seed orchard	Local Stand				
Block	East	South	West	North	Average
BI/3	0.291±0.19	0.263±0.14	0.090±0.20	0.335±0.16	0.392±0.29
BIV/4	0.245±0.16	-0.080±0.12	0.272±0.12	0.138±0.13	0.505±0.21
BVI/5	0.244±0.15	-0.103±0.10	0.142±0.11	0.249±0.12	0.220±0.17
Average	0.308±0.12	-0.217±0.08	0.103±0.10	0.206±0.09	0.297±0.18

Estimates of observed and expected heterozygosity (Ho, He), Wright's fixation index (F), and Chi-square (χ^2) test of Hardy-Weinberg equilibrium for the six loci at each local stand and each block in the seed orchard are presented in Table 3.5. The fixation index ranged from –0.0396 (locus *Ak-1*, sector I block 3) to 1.00 (*Idh-2* and *Mdh-2* in all local stands and seed orchard blocks). The Chi-

Table 3.5. Wright's Fixation Index (F), observed (Ho) and expected (He) heterozygosities at single-loci.

Population		Gene locus	Ho	He	F	χ^2 (df)
Local stand	West	*Ak-1*	0.030	0.029	-0.015	0.039 (1)
		Idh-2	0.000	0.232	1.000	426.1 (3)**
		Mdh-2	0.000	0.263	1.000	202.9 (1)**
		Pgm-1	0.125	0.224	0.440	41.8 (3)**
		Pgm-3	0.200	0.440	0.544	59.9 (1)**
		Skdh-1	0.200	0.415	0.517	102.0 (3)**
	North	*Ak-1*	0.015	0.015	0.0076	0.008 (1)
		Idh-2 *	0.000	0.000	-	-
		Mdh-2	0.000	0.296	1.000	202.4 (1)**
		Pgm-1	0.035	0.054	0.346	26.5 (1)**
		Pgm-3	0.190	0.443	0.570	65.7 (1)**
		Skdh-1	0.185	0.423	0.562	94.1 (3)**
	South	*Ak-1* *)	0.000	0.000	-	-
		Idh-2	0.000	0.432	1.000	429.5 (3)**
		Mdh-2	0.000	0.425	1.000	201.4 (1)**
		Pgm-1	0.125	0.274	0.543	455.9 (3)**
		Pgm-3	0.180	0.376	0.520	54.8 (1)**
		Skdh-1	0.150	0.519	0.710	143.7 (3)**
	East	*Ak-1*	0.075	0.072	-0.039	0.028 (1)**
		Idh-2	0.000	0.249	1.000	203.0 (1)**
		Mdh-2	0.000	0.256	1.000	202 .1 (1)**
		Pgm-1	0.160	0.232	0.310	22.0 (3)**
		Pgm-3	0.285	0.427	0.331	22.26 (1)**
		Skdh-1	0.260	0.540	0.517	104.8 (3)**
Seed orchard	Block 3/sector I	*Ak-1*	0.113	0.108	-0.040	1.388 (6)
		Idh-2	0.000	0.340	1.000	814.3 (3)**
		Mdh-2	0.000	0.276	1.000	402.6 (1)**
		Pgm-1	0.258	0.278	0.074	57.7 (3)**
		Pgm-3	0.270	0.439	0.385	59.6 (1)**
		Skdh-1	0.125	0.259	0.516	195.6 (3)**
	Block 4/sector III	*Ak-1*	0.083	0.080	-0.038	0.717 (6)
		Idh-2	0.000	0.266	1.000	820.6 (3)**
		Mdh-2	0.000	0.238	1.000	403.2 (1)**
		Pgm-1	0.083	0.122	0.320	132.1 (3)**
		Pgm-3	0.175	0.454	0.614	151.5 (1)**
		Skdh-1	0.25	0.537	0.534	243.5 (3)**
	Block 5/sector VI	*Ak-1*	0.025	0.025	-0.013	0.058 (1)
		Idh-2	0.000	0.266	1.000	820.6 (3)**
		Mdh-2	0.000	0.255	1.000	402.9 (1)**
		Pgm-1	0.095	0.117	0.189	14.8 (1)**
		Pgm-3	0.310	0.498	0.377	57.2 (1)**
		Skdh-1	0.258	0.604	0.573	277.8 (3)**

* Denotes monomorphic locus

** Denotes significant departure from Hardy Weinberg at 0.01 level

square test on the fixation index rates indicated a significant excess of homozygotes in all stands and blocks for all loci except for *Ak-1*.

Estimates of single-locus and multilocus outcrossing rates with the standard errors based on 200 bootstraps for each local stand and seed orchard block are presented in Table 3.6. Single-locus estimates of outcrossing rates (t_s) varied from 0.102 to 0.244. The multilocus estimates of outcrossing rates (t_m) ranged from 0.137 to 0.305.

Table 3.6. Estimate of single-locus (t_s) and multilocus (t_m) outcrossing rates, and their standard error.

	Population	t_s	t_m
Local stand	North	0.244 ± 0.095	0.305 ± 0.100
	East	0.215 ± 0.094	0.274 ± 0.107
	South	0.153 ± 0.087	0.281 ± 0.05
	West	0.214 ± 0.086	0.290 ± 0.084
Seed orchard	Block 3 (sector I)	0.102 ± 0.039	0.137 ± 0.052
	Block 4 (sector IV)	0.200 ± 0.070	0.238 ± 0.064
	Block 5 (sector VI)	0.208 ± 0.057	0.252 ± 0.640

DISCUSSION

Factors such as flowering synchronization, pollen production and distribution, wind direction, isolation zone tree species, and some technical problems combined could possibly contribute to the substantial level of contamination reported here.

P. merkusii native to Sumatra initiates flowers throughout the year (Cooling 1968). Moreover, for this orchard, in which the trees were derived from different elevations and regions all over Java, the varied source environments could also cause variable timing of flowering activity among the families. This is one of the assumptions that was violated for this seed orchard and could cause difficulty in reaching genetic efficiency. Well-matched flowering periods among orchard trees is very important to reduce contamination. Lack of synchrony of

flowering period among trees within the orchard and the coincidence of some female flowers in the orchard with the time of pollen shedding from trees outside resulted in the overall contamination rate being quite high.

Even though the orchard trees were old enough to produce sufficient pollen for fertilizing the orchard trees, the proportion of background pollen was probably still very high especially from the old stand. Besides, the distribution of the pollen release all over the orchard is also very important in minimizing contamination. These factors, together with wind direction resulted in the high level of contamination in this seed orchard. The results presented in Table 3.4 showed that the pollen contamination rates on average over blocks were significantly higher coming from the east and north sources. This could be due to the prevailing wind direction (northeast), and the fecundity of the east stand (an old pine plantation from 1962 and 1977). The trees from this stand might have produced large amounts of pollen that dispersed into the orchard and successfully accomplished fertilization when there was not sufficient quantity of well distributed seed orchard pollen.

The isolation zone trees were younger than the seed orchard trees, and most of them had not grown as high as the *P. merkusii* trees inside the seed orchard. Lack of barrier in the isolation zones, coupled with the typical habit of female flowers being borne in the upper crown of the pine trees might also permit pollen contamination.

The $\chi 2$ test showed a significant deficiency of heterozygotes in both the seed orchard and unselected background stands. The positive values of the inbreeding coefficient (F) indicated a lower frequency of the observed heterozygotes than expected under Hardy Weinberg equilibrium. The excess of homozygotes in this orchard complex suggests strong inbreeding, hence a further analysis on the single-locus and multilocus outcrossing rates was needed. A negative value for F was found only at locus *Ak-1* in all stands and seed orchard blocks, and the $\chi 2$ test revealed an insignificant excess of homozygosity. Two loci *Idh-2* and *Mdh-2* had F = 1, since there were no heterozygotes found in the embryos for these loci.

The estimation of the outcrossing rates shows a high level of selfing in all local stands and blocks. The mode of mating in this seed orchard complex could be classified as mixed selfing and outcrossing (Brown 1990), with a very low level of the multilocus outcrossing rates (t_m) that ranged from 0.137 to 0.305, and single-locus outcrossing rates (ts) ranged from 0.102 to 0.244. All of the estimates of the multilocus outcrossing rates are higher than single-locus rates, indicating that most or all apparent selfing is a result of consanguineous mating, not actual self-fertilization (Shaw and Allard 1982).

The extremely high inbreeding coefficient, and thus the significant excess of homozygotes, and extraordinary low outcrossing rates in this orchard complex may be due to the layout of the seed orchard and the origin of the trees. After several roguings, there were one or two trees per family left on the site that were laid out in a family row (when the samples were collected). The planting positions of some progenies of the same family were adjacent to each other, and the families/progenies planted are replicated in each block, and are also replanted in the adjacent sector. This design could enable crosses between progeny of the same families.

The local stands could possibly consist of trees that were derived from the same sources or even from the seed orchard itself, thus they could be genetically related to the orchard trees, and therefore every successful fertilization from these trees would be a consanguineous mating product.

Inbreeding depression caused by selfing could result in non-viable embryos due to homozygosity of lethal recessive genes, abnormal seedling, weak plant growth, or low seed viability (Hadders and Koski 1975, Khasa et al. 1993). The inbreeding depression caused by consanguineous mating is not as severe as self-fertilization, but its consequences may be more critical for reforestation programs. The mildly inbred seedlings produced by consanguineous matings may survive past the seedling stage, but would still suffer from a loss of heterozygosity, which could lead to inferior growth and productivity as mature trees (Ritland and El-Kassaby 1985)

Several technical factors could also influence the accuracy of the estimation of the contamination level, and the estimation of mating system parameters. In this study they could include the enzyme system used, skipping some of the families, and errors in genotyping of the sample (Paule 1991).

MANAGEMENT CONSIDERATIONS

The production of genetically improved seed is a major goal of any tree improvement program. When pollen contamination and inbreeding take place in a seed orchard, the seed produced is obviously of uncertain genetic merit. Some management techniques that reduce pollen contamination and minimize inbreeding should be considered for this orchard. These include:

Widening the isolation zone

The results revealed that contaminant pollen could travel a long distance to the orchard. A wider isolation zone is suggested for this seed orchard or conversion of the surrounding *P. merkusii* plantations into plantations of other species especially on the windward side. In wind pollinated seed orchards, Adams et al (1991) have reported that without extensive spatial isolation (e.g. 1.6 km or more) from non-orchard pollen sources, levels of contamination can be quite high.

Amelioration

Amelioration involves planting the area surrounding the seed orchard with families of the same species that perform well. This will help offset the bad effect of contamination even though it might not reduce contamination (Lowe and Wheeler, 1993).

Studies on flowering of P. merkusii

Detailed studies about phenological patterns of *P. merkusii* families comprising seed orchards and background stands may be necessary. This is a fundamental basis for any techniques applied to reduce pollen contamination and

inbreeding in the orchard, such as supplemental mass pollination (SMP), flower stimulation, girdling to induce flowering, bloom delay (cooling treatment to delay bud burst using sprinklers). SMP may be a potential way to improve the genetic worth of seed crops. The concepts have been reviewed recently by Webber (1995).

Controlled pollination

The last possible solution to avoid contamination and inbreeding is to convert the orchard from an open-pollinated seed orchard into a controlled pollination seed orchard. This has happened in New Zealand, where a radiata pine (*Pinus radiata* D Don) seed orchard was converted from open-pollinated into a controlled-pollinated seed orchard in 1984 (Sweet, 1995).

Continual test on mating system and pollen contamination

Environmental factors, such as humidity, wind speed, wind direction, ground cover, and crown influence pollen release and distribution, and are prerequisites for the success of fertilization (Wheeler et al. 1993). Thus, pollen contamination and mating system depend on the same environmental factors. Because of the variability of the environmental factors throughout the year, the level of pollen contamination, mating system parameters and the genetic composition of seed orchard seed may also fluctuate. Continual testing of pollen contamination and mating system are probably necessary to determine the best time to harvest the seed orchard seed, not just when the seeds are ripe but also when the rate of contamination is acceptable with minimum inbreeding.

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CHAPTER 4

GENETIC DIVERSITY OF *Pinus merkusii* Jungh. & De Vriese IN A SEEDLING SEED ORCHARD AND IN ITS SURROUNDING STANDS AS DETECTED BY ALLOZYME MARKERS

INTRODUCTION

Biological diversity is defined by U.S. Government's Office of Technology as the variability among living organisms and the ecological complexes in which they occur (Skroppa 1994). It is the most fundamental concept in ecology, population biology and population genetics (Gregorius 1987).

The term biological diversity encompasses three different levels (Skroppa 1994): ecological diversity, characterizing variation patterns between and within ecosystems; species diversity, characterizing the number of species and their distributions; and genetic diversity, characterizing the genetic variability between and within populations of a single species.

The amount and kind of genetic variability in forest tree populations are potentially affected by evolutionary processes (migration, mutation, genetic drift, and selection), mating system (Lewontin 1982), and human activities in forest management over centuries (El-Kassaby and Namkoong 1994, Skroppa 1994)

Various modes of forest management practices potentially impact the magnitudes of the evolutionary processes, thus influencing the level of genetic diversity of populations. Forest practices vary from natural regeneration to intensive industrial forestry, including breeding programs, harvesting methods (selective cutting, clear-cutting), and sylvicultural activities. These practices may result in unintentional and intentional changes in gene pools, or may cause alterations in the mating system, which, in turn, influence the level of inbreeding (Savolainen and Karkkainen 1992, El-Kassaby and Namkoong 1994).

Some studies have been carried out on the impact of forest management practices in shaping the genetic structure of forest tree populations using isozyme loci. Buchert et al (1997) examined the effect of harvesting on genetic

diversity in eastern white pine, and they found real and repeatable genetic erosion occurred in the gene pools as a result of harvesting. Chaisurisri and El-Kassaby (1994) compared the genetic diversity between seed production populations and natural populations of Sitka spruce, and they observed that the orchard population was significantly higher in number of alleles per locus, percentage of polymorphic loci and heterozygosity. Muona and Harju (1989) studied seed orchard and natural populations of *Pinus sylvestris*, Cheliak et al. (1988) evaluated the genetic effects of phenotypic selection in white spruce, and many other studies could be mentioned.

The degree of genetic diversity is the key concept in determining a strategy in sampling design for genetic conservation or a tree improvement program (Yeh 1989, Liengsiri et al. 1990). The maintenance of high levels of genetic diversity in forest tree populations is essential for adaptability, productivity (Millar and Westfall 1992), viability, survival, and evolution (Hamrick and Godt 1981, Ledig 1988).

Seedling seed orchards, the deployment tool of many breeding programs, are established through plantations of plus trees selected from plantations or natural stands based on traits of interest such as volume growth and stem and branch quality traits. If the selection is effective, then the seed orchard population may be different in its genetic structure and genetic diversity from the original populations, usually less variable (Skroppa 1994). And since a seed orchard usually consists of a restricted number of genotypes compared to natural populations, a risk of loss of genetic variability in the future plantation has become a big concern for geneticists (Szmidt, 1987, Wheeler and Jech 1992, Chaisurisri and El-Kassaby 1993, El-Kassaby and Namkoong 1995). However, this concern does not appear to be valid, at least with respect to genetic variation at the allozyme level. That is, seed orchards retain nearly as much as genetic diversity as natural populations (reviewed in Wheeler and Jech 1992) even when population sizes are as low as 25-30 clones.

Isozyme analysis has been used in many aspects of forest genetics. Samples of megagametophytes, embryos, pollen, buds or needles of trees are

crushed, and absorbed onto filter paper wicks that are placed in a starch or polyacrylamide gel, and exposed to an electrical current. These isozyme techniques (Friedman and Neale 1993) provide genetic markers for estimating levels of genetic variation in natural and artificial populations of forest trees, mating systems, pollen neighborhood, progeny variability, population structure and seed dispersal (Loveless 1992). Allozyme markers are also useful in other aspects of forest genetic conservation such as determining sampling plans for in-situ and ex-situ conservation, and monitoring change in genetic diversity (Millar and Westfall 1992)

Starch gel electrophoresis of isozymes has been used in elucidating patterns of population variation in many conifers; it provides a faster assessment of genetic variation than the traditional approach, which requires a series of controlled crosses; and a great number of gene loci can be studied (Chaisurisri and El-Kassaby 1994).

In this study, isozyme gene markers were employed to analyze genetic variation of *P. merkusii* in a seedling seed orchard and adjacent local stands, in east Java, Indonesia. We examined the genetic diversity existing in different blocks in the seed orchard, and in different stands surrounding it, and also the difference between the orchard and local stand in their genetic composition.

MATERIAL AND METHODS

Seed collection

The orchard is an open-pollinated seedling seed orchard located in Forest District Jember, Sub Forest District Sempolan, East Java-Indonesia, and is owed by Perum Perhutani (State Forest Company).

The seed orchard covers an area of 96 ha, and consists of plus trees that were selected from *P. merkusii* plantations all over Java. The area is divided into six sectors, representing the planting year (1977-1982); each sector consists of 10 blocks that were arranged in a completely randomized block design. In the initial planting, each sector was composed of 200 families that consisted of 160

new selected families along with 40 families that were already tested in the previous year in the previous sector. Each family consisted of 5 seedlings (Suseno 1984). Roguing has been done several times to eliminate the poor families/trees, causing the distance between trees to be irregular. However data on the number of families and trees left were not available.

The orchard was surrounded by a 200-meter wide isolation strip, which was composed of a plantation of different species of different ages. Beyond the isolation zone there were forest plantations of different forest tree species of various ages, but mainly *P. merkusii* that were planted for wood and resin production.

Cones were collected from both genetically improved *P. merkusii* trees in the seed orchard, and from the planted *P. merkusii* in the stands surrounding the orchard (Fig. 3.1, Chapter 3). The sample collection was carried out in August and September 1997.

From inside the orchard, cones were obtained from trees in three different blocks that were randomly sampled from different years of plantation as shown in Fig. 3.1 (Chapter 3). They were from sector I plantation (1978) block 3 (I/3); sector IV plantation (1981) block 4 (IV/4); and sector VI plantation (1983) block 5 (VI/5). Forty trees that represent forty families were sampled per block.

From the area surrounding the orchard, 20 pine trees were sampled from each of forest stands No.38e, No. 40e, No. 49a, and No. 29e, which were located west, east, north, and south of the orchard, respectively. These background stands were pine plantations established in 1979, 1962, 1992, and 1981 for the west, east, north, and south respectively (Fig. 3.1, Chapter 3).

The cones were air-dried under sunlight, separated by family, and then the seeds were extracted, cleaned by hand and stored at 4 ^{o} C until electrophoretic analyses at the University of Alberta.

Electrophoretic analyses of genetic diversity.

The genotype of each individual sample tree was inferred for 20 loci that were consistently and clearly scored from megagametophyte and embryo tissues (Suwarni et al. 1999). Ten seeds per tree were electrophoretically assayed. The probability of correctly identifying a heterozygote at a particular locus is $1-(1/2)^{k-1}$, for k megagametopytes assayed per tree. With 10 seeds assayed per tree, the probability of miss-classifying a heterozygote is 0.195 %.

The 20 loci were encoded from nine enzyme systems that were run using the H buffer system (Suwarni et al. 1999). The nine enzymes were isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC. 1.1.1.37), phosphoglucomutase (PGM, EC. 5.4.2.2), shikimate dehydrogenase (SKDH, EC. 1.1.1.25), adenilate kinase (AK; EC.2.7.4.3). aconitase (ACO, EC.4.2.13), alcohol dehydrogenase (ADH, EC.1.1.1.1), malic enzyme (ME, EC. 1.1.1.40), 6-phosphogluconic dehydrogenase (6-PGD, EC.1.1.1.44). The electrophoresis of isozyme procedures, and the inheritance of allozymes variants have been described elsewhere (Suwarni et al. 1999).

Genetic parameters

Genetic variability of the 120 trees inside the orchard and the 80 trees from the local stands surrounding the orchard were determined based on the loci and allele banding patterns in individual embryos. Both monomorphic loci and polymorphic loci were used in this analysis.

Several types of allelic and genotypic diversity parameters were calculated to describe levels of genetic variability in both the seed orchard and the local stand populations. The parameters were: percent of polymorphic loci (P), mean number of alleles per locus (n_a), effective number of alleles per locus (n_e), and expected genetic diversity or heterozygosity (*He*) (Hamrick et al 1992, Hattemar 1991, Nei 1987, Weir 1989). In this study, the local stand and seed orchard block

was considered as population level and the total measure was obtained by pooling the blocks and the surrounding stands.

The average percentage of polymorphic loci within populations (P_p) is the proportion of loci polymorphic in each population averaged over all populations. A gene locus was considered polymorphic only if the frequency of the most common allele was equal to or less than 95 %.

The genetic diversity per locus within populations was calculated using the formula:

$$He_p = 1 - \sum x_i^2$$

where x_i is the frequency of the i allele in each population. Average heterozygosity or genetic diversity of each locus was obtained by averaging over all populations and over all loci.

The mean number of alleles per locus within populations (n_{ap}) was determined for each population and a mean value obtained by averaging over all populations. The effective number of alleles within populations (n_{ep}) was calculated using formula by Hamrick et al (1992):

$$n_{ep} = 1/(1 - He_p)$$

Another genetic diversity concept that was also considered in this study was allelic diversity. Allelic diversity refers to the number and distribution of allelic variants that exist in the population. It reflects the potential of the population to produce different genotypes and expresses its evolutionary potential (Skroppa 1994).

All of the measurements of the genetic variation parameters were performed by the computer program POPGENE version 1.21 computer program of Yeh et al. (1997).

RESULTS

The seed orchard and the adjacent local stands had similar alleles at each locus studied. Of the 20 loci examined from the embryo tissues, six loci were found to be polymorphic at the 95 % criterion. The percentage of polymorphic loci (P) was observed to be the same in each of the orchard blocks and the local stands (30 %), except for the north stand, which contained fewer polymorphic loci (25 %). For pooled populations, the number of alleles per locus (n_a), the effective number of alleles per locus (n_e), and the total genetic diversity (*He*) were 1.75,1.14, and 0.088, respectively (Table 4.1).

Table 4.1 Observed number of alleles per locus (n_a), effective number of alleles per locus (n_e), percentage of polymorphic loci (P), and observed and expected heterozygosity (*Ho*, *He*) of *P. merkusii* in the seed orchard and in surrounding stands.

Sample	P (95%)	n_a (se)*	n_e (se)	*Ho* (se)	*He* (se)
Block 3/ sector I	30	1.65	1.13	0.039	0.085
Block 4/sector IV	30	1.60	1.14	0.030	0.085
Block 5/sector VI	30	1.45	1.17	0.035	0.089
Average	30	1.60 (0.085)	1.15(0.017)	0.035(0.004)	0.086(0.002)
East	30	1.40	1.15	0.039	0.089
North	25	1.30	1.10	0.021	0.061
South	30	1.50	1.18	0.024	0.102
West	30	1.45	1.12	0.028	0.080
Average	28.75	1.41	1.14	0.028	0.083
Pooled populations	30	1.75(0.014)	1.14(0.005)	0.032(0.001)	0.088(0.003)

*se = standard error

The percentage of polymorphic loci (P_p) in the seed orchard was slightly higher (30 %) than that in the surrounding stands (28.75 %). The observed number of alleles per locus (n_{ap}) and the effective number of alleles per locus (n_{ep}) in the seed orchard were not significantly different from those in the local stands. The n_{ap} and n_{ep} values were 1.60 and 1.15, respectively, in the seed orchard, and 1.41 and 1.14 in the surrounding stands. Two alleles of *Ak-1* were

present only in the seed orchard in low frequency: 0.0071 and 0.0054, respectively, for allele *Ak-1*-D and *Ak-1*-F. Two other alleles (*Adh-1*- B and *Me-2*-B) were also present only in the seed orchard at very low frequency: 0.0004 for each of them, although allele B of locus *Idh-1* was only found in the local stand. The mean allozyme frequencies of *P. merkusii* in the seed orchard and in the surrounding plantation for all allozyme loci are presented in Table 4.2.

Table 4.2 The mean allele frequency of *P. merkusii* in the orchard and the surrounding stands

Locus	Allele	Seed Orchard	Local Stand	Locus	Allele	Seed Orchard	Local Stand
AK-1	A	0.9633	0.9850	*Pgm-1*	A	0.9033	0.8875
	B	0.0242	0.0150		C	0.0829	0.1087
	D	0.0071			E	0.0138	0.0037
	F	0.0054		*Pgm-2*	A	1.0000	1.0000
Ak-2	A	1.0000	1.0000	*Pgm-3*	A	0.6208	0.6969
Ak-3	A	1.0000	1.0000		B	0.3792	0.3031
Idh-1	A	1.0000	0.9994	*SKdh-1*	A	0.6679	0.6687
	B		0.0006		B	0.1613	0.2362
Idh-2	A	0.8300	0.8562		C	0.1708	0.0950
	B	0.0342	0.0112	*Skdh-2*	A	1.0000	1.0000
	C	0.1358	0.1325	*Skdh-3*	A	1.0000	1.0000
Mdh-1	A	1.0000	1.0000	*Adh-1*	A	0.9996	1.0000
Mdh-2	A	0.8492	0.8025		B	0.0004	
	B	0.1508	0.1975	*Aco*	A	1.0000	1.0000
Mdh-3	A	1.0000	1.0000				
Me-1	A	1.0000	1.0000	*6Pgd-1*	A	0.9975	0.9988
Me-2	A	0.9996	1.0000		B	0.0025	0.0013
	B	0.0004		*6Pgd-2*	A	1.0000	1.0000

The average observed (*Ho*) and expected heterozygosity (*He*) measure of Nei (1987) in the seed orchard were 0.035 and 0.086, respectively, and 0.028 and 0.083 in the local stand population. A summary of genetic diversity measures at six polymorphic loci with their standard errors is given in Table 4.1.

DISCUSSION

The results of this study provide evidence that *P. merkusii* in this seedling seed orchard complex (in Java) has relatively low genetic diversity compared to other pines in the world. As reviewed by Changtragoon and Finkeldey (1995), most pines showed genetic diversity of isozyme loci usually being higher than 0.15, whereas the genetic diversity observed in this study was 0.088.

However compared to natural populations in Thailand, the level of genetic variation of *P. merkusii* in this seed orchard complex is slightly higher. The measures in this study were 30 % (*P*), 1.75 (n_a) and 0.088 (*He*), while in natural stands in Thailand; they were 18.8 % (P), 1.6 (n_a) and 0.058 (*He*) (Changtragoon and Finkeldey 1995).

Compared to the study of Szmidt et al (1996) of *P. merkusii* in natural populations in Thailand and Vietnam, the species in Java was also found to be more diverse. The observed heterozygosity (*Ho*) and expected heterozygosity (*He*) in Thailand and Vietnam populations ranged from 0.016 to 0.075, and 0.019 to 0.083, respectively. In this study we observed the genetic variation of *P. merkusii* ranged from 0.031 to 0.033 for observed heterozygosity, and from 0.085 to 0.091 for expected heterozygosity. This indicated that the *P. merkusii* in this study harbored more variation than *P. merkusii* in Thailand and Vietnam provenances.

Besides the differences in the heterozygosity level, differences were also observed in allozyme frequencies for some loci. For example, *Pgm-1* appeared to be invariant in *P. merkusii* from Thailand and Vietnam provenances (Szmidt et al 1992), while in this seed orchard complex we observed three alleles with the frequencies 0.9033, 0.0829 and 0.0138 in the seed orchard, and 0.8875, 0.1087 and 0.0037 in the local stands (Table 4.2). *Pgm-2* was found to be invariant in our study, but in Thailand and Vietnam it was observed to have two alleles with a frequency of 0.006 and 0.994. *Mdh-1* had two alleles in their study, whereas no variation was observed for this locus in our study.

The genetic differences presented above are similar to differences existing in some morphological and biochemical characteristics between the southeast

Asia mainland provenance and the Sumatran provenance, which is the original source of pine trees in Java (Cooling 1968). This variation includes: seed weight, presence or absence of a grass-stage in the seedlings, nodal habit, needle and cone dimensions, wood density patterns, oleoresin constituents, tree size and bole form, habitat and natural range (Cooling 1968). The greater genetic diversity and the presence of rare allelic variants in Sumatran populations of *P. merkusii* would suggest Sumatra might be the center of diversity of *P. merkusii.*

The *P. merkusii* trees studied in this seedling seed orchard were derived from superior trees that were selected from plantation forests all over Java, and the local plantations were also from some areas in Java. The planted *P. merkusii* in Java was originally from the Sumatran natural population. The Sumatran population is spread over 3 different locations: *Aceh, Tapanuli* and *Kerinci* and the *P. merkusii* plantations in Java were almost exclusively formed from the *Aceh* race (Cooling 1968). So far we don't have any information about the allozyme genetic structure of *P. merkusii* from Sumatra, nor do we know about the evolutionary processes that influenced its genetic structure

The *P. merkusii* plantation in Java is a very well managed plantation that has been exposed to many kind of aspect forest management activities such as sylvicultural treatments, harvesting and regeneration methods. These kinds of forest management activities might also play an important roles in shaping the genetic structure of the pine forest in Java, and thus of the pine trees in the orchard studied, as they came from plantations in Java.

Artificial regeneration has the potential to affect the genetic composition of the forest stand because the species and composition can be manipulated and controlled (El-Kassaby and Namkoong 1994). Seed sowing in the nurseries, which is needed for artificial regeneration has the potential to influence genetic diversity through directional selection. It is a common practice in nurseries to eliminate the small germinants, and leave the largest ones, which are generally the earliest germinants. This practice introduces a strong selection pressure favoring the faster germinating seed parents (El-Kassaby 1992).

Stand tending, a sylvicultural treatment that aims to stimulate the growth of the remaining trees in a forest stand, could influence the genetic structure of a forest population (El-Kassaby and Namkoong 1994). This is usually accomplished by removing some trees (the smaller or infested trees) from the stand during its development. In Java these treatments are carried out at the age of 5, 10, and 15 years old in *Pinus merkusii*. This practice can be in the form of spacing or thinning, which both could affect stand genetics by controlling species composition, providing the opportunity for phenotypic selection with great selection intensity, and could reduce family structure (El-Kassaby and Namkoong 1994).

The mating system and the breeding program that is still underway in this seed orchard might play an important role in determining population structure. Since regeneration of *P. merkusii* in Indonesia relies on sexual reproduction (i.e. seeds), there is a large possibility that mating system plays an important role in shaping the genetic variation of the *P. merkusii* in this seed orchard complex.

Comparing the measures of the genetic parameters of the *P. merkusii* in the seedling seed orchard with those in the surrounding plantations indicated that slightly more genetic variability was observed in the seed orchard than that in the surrounding stands. This might be due to the broader genetic base of the seed orchard genotypes, as they were derived from a very wide area (all over Java), whereas the local stands might have been derived from a smaller range of sources in Java or from the seed orchard itself.

MANAGEMENT IMPLICATION

For a successful long-term tree improvement program, it is essential to begin with a broad genetic base and to use a breeding program that will conserve genetic potential that is already in the population (Zobel and Talbert 1984). Therefore it is necessary to study the genetic variation existing in natural populations of *P. merkusii* (i.e. Sumatra), and compare it with the plantations in Java as well as with *P. merkusii* populations in this orchard. This is to ensure that

the *P. merkusii* in the seed orchard consists of a broad genetic diversity, and all of the genetic variation of *P. merkusii* in the natural distributions was captured. Because a seed orchard is a major source of genetic material for future forests (El-Kassaby and Ritland 1996), the genetic diversity in a seed orchard should be maintained, at least at the level as that in the natural populations (Chaisurisri and El-Kassaby 1994).

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CHAPTER 5

GENERAL CONCLUSIONS

RECAPITULATION OF IMPORTANT FINDINGS

Pinus merkusii Jungh. & De Vriese, a tropical pine species that is native to Sumatra, is widely planted in Indonesia and is one of the priority species for tree improvement. Information on the mating system, pollen contamination and genetic diversity of this species is essential for the success of the tree improvement programs and gene conservation.

The study has been completed in examining the proportion of pollen contamination, mating system parameters, and genetic diversity of *Pinus merkusii* in a seedling seed orchard complex in Forest District Jember, East Java, Indonesia, using allozyme variants. Prior to this study the allozyme variants of the haploid (1 N) megagametophyte and the diploid (2N) embryo tissues of *P. merkusii* seed was examined to establish reliable models of their inheritance. Five out of 20 isozyme loci from nine enzyme assayed were found to be polymorphic at the 95 % criterion in both megagametophyte and embryo tissues. They include *Idh-2, Mdh-2, Pgm-1, Pgm-3* and *Skdh-1*. All polymorphic loci of the haploid megagametophyte tissues had alleles that segregated following expected Mendelian frequencies, except for *Pgm-1* with 100/81 allelic combination showing significant segregation distortion. The remaining loci were monomorphic (*Aco, Ak-2, Ak-3, Mdh-1, Mdh-3, Me-1, 6Pgd-2, Pgm-2, Skdh-2, Skdh-3*), or nearly monomorphic with rare alleles in heterozygous state for *Adh-3, Ak-1, 6Pgd-1, Idh-1* and *Me-2*.

Both the megagametophyte and embryo tissues were assayed and genotyped to identify the contribution of the maternal and the paternal parents to the embryo. The amount of pollen contamination and mating system parameters in the *Pinus merkusii* seedling seed orchard were estimated using the genotypic data from the embryo tissues. The estimation is based on the single locus allelic frequency differences between the seed orchard population and the plantation of

the species surrounding the orchard. The study revealed a substantial level of contamination in the seed orchard, 29.7% ± 18%. A significant excess of homozygotes obtained from this orchard complex suggests that consanguineous mating have occurred. The outcrossing rates were very low and varied over blocks and local stands. Multilocus outcrossing rates (t_m) ranged from 0.14 to 0.31, and single locus outcrossing rates (t_s) ranged from 0.10 to 0.24. Factors such as seed orchard design, wind direction, flowering synchronization, isolation trees, and technical aspects could contribute to this level of pollen contamination and inbreeding. Some management considerations like widening the isolation zone or intensive pollen management (e.g. supplemental mass pollination and bloom delay) are the best possible solutions for this seed orchard.

The measures of genetic variation of this species in the seed orchard, and in the plantation stands surrounding the orchard, indicated that this species had a relatively low level of genetic diversity compared to other conifers. However compared to natural populations in Thailand, the level of genetic variation of *P. merkusii* in this seed orchard complex is somewhat higher. The measures in this study were 30 % (*P*), 1.75 (n_a) and 0.088 (*He*), while in natural stand in Thailand; they were 18.8 % (P), 1.6 (n_a) and 0.058 (*He*).

The gene diversity did not differ significantly between the seed orchard and the surrounding stands. The average percentage of polymorphic loci (95 % criterion) was 30 % and 28.5 % for the seed orchard and local stands, respectively. The observed number of alleles per locus, the mean effective number of alleles per locus, and the expected heterozygosity were 1.60, 1.15, and 0.086 in the seed orchard and 1.41, 1.14, and 0.083 in the local stands, respectively.

POSSIBLE RESEARCH FOR THE FUTURE

Allozyme markers can detect variation only for protein-coding genes, and only a fraction of all mutational events (i.e. those changing protein mobility in a gel) can be resolved, and are also subject to ontogenetic variation. DNA-based

markers overcome these disadvantages. Therefore, DNA markers are becoming more popular in genetic studies such as those conducted in this thesis. Of particular importance are the cpDNA markers that can be useful for monitoring pollen contamination in seed orchards. The accuracy of mating system and genetic diversity studies will be enhanced by using hypervaluable and codominant markers such as microsatellites.

It is of great importance to continually investigate pollen contamination and mating system in order determine the best time to harvest seed, not just when the seeds are ripe but also when the rate of contamination is acceptable with minimum inbreeding in this seedling seed orchard.

Detailed studies about phenological patterns would be necessary for maximizing synchronization of flowering among seed orchard trees and for any cultural treatment to be applied to this orchard to reduce pollen contamination and inbreeding.

Further research on genetic variation existing in natural populations of *P. merkusii* (i.e. in Sumatra), and in plantation populations in Java, including seed orchards will be beneficial for successful tree improvement programs and genetic conservation.

APPENDICES

Appendix 1

Enzyme staining recipes, storage condition of buffer solutions and chemicals

Aspartate aminotransferase (AAT) EC 2.6.1.1

Phyridoxal-5-phosphate	0.002	g
Fast blue BB salt	0.05	g
AAT substrate soulution	25	ml

Incubate in the dark 37 ° C until dark blue bands appear

Aconitase (ACO) E.C. 4.2.1.3

0.2 M Tris-HCl, pH 8.0	25.0	ml
5 % cis-aconitic acid, pH 8.0	2,5	ml
Isocitric dehydrogenase	20	units
1 % $MgCl_2$	0.5	ml
NADP	0.5	ml
NBT	0.5	ml
PMS	0.5	ml

Incubate in the dark 37 ° C until dark blue bands appear

Adenilate Kinase (AK) E.C.2.7.4.3

0.2 M Tris-HCl, pH 8.0	25.0	ml
Glocose	50	mg
ADP	50	units
Hexokinase	125	units
Glucose-6-phosphate dehydrogenase	25	units
1 % $MgCl_2$	0.5	ml
NADP	0.5	ml
MTT	0.5	ml
PMS	0.5	ml

Incubate in the dark about 120 min. at 37 ° C until dark blue bands appear

Alcohol dehydrogenase (ADH) E.C. 2.7.5.1

0.2 M Tris-HCl, pH 8.0	25	ml
Alcohol 95 %	0.5	ml
1 % $MgCl_2$	0.5	ml
NADP	0.5	ml
MTT	0.5	ml

PMS	0.5 ml

Incubate in the dark at 37 ° C until dark blue bands appear

Esterase (Colorometric) EC 3.1.1.1

0.2 M Phosphate buffer, pH 6.4	25 ml
α and β - naphthyl acetate in 2.5 acetone	25 mg
Fast blue RR salt	50 mg

Isocitric dehydrogenase (IDH) E.C. 1.1.1. 4.2.

0.2 M Tris-HCl, pH 8.0	25 ml
DL-isocitric acid, trisodium salt	100 mg
1 % $MgCl_2$	0.5 ml
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

Incubate in the dark about 60 min. at 37 ° C until dark blue bands appear

Leucine-amino peptidase (LAP) EC 3.4.11.1

(a) 0.5 boric acid	50 ml
(b) 0.2 M Tris-HCl, pH 8.0	25 ml
Tris-maleic, pH 5.3	25 ml
10 % MgCl2	0.5 ml
L-leucine β-naphthylamide-HCl	25 mg
Fast black K salt	35 mg

Soak gel in boric acid for 15 min., empty solution, and rinse gel with distilled water then stain the gel with the mixed solution (b)

Incubate in the dark about 60 min. at 37 ° C until dark blue bands appear

Malate dehydrogenase (MDH) E.C. 1.1.1.37

0.2 M Tris-HCl, pH 8.0	12.5 ml
DL-malic acid, pH 7.0	12.5 ml
NAD	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

Incubate in the dark about 45 min. at 37 ° C until dark blue bands appear

Malic Enzyme (ME) E.C. 1.1.1.40

Electrode buffer H	12.5 ml
0.5 DL-malic acid, pH 7.0	2.5 ml
1 % $MgCl_2$	0.5 ml
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

Incubate in the dark at 37 ° C until dark blue bands appear

6-phosphogluconate dehydrogenase (6-PGD) E.C. 1.1.1. 44

0.2 M Tris-HCl, pH 8.0	25 ml
6-phosphogluconic acid, trisodium salt	15 mg
1 % $MgCl_2$	0.5 ml
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

Incubate in the dark about 45 min. at 37 ° C until dark blue bands appear

Phosphoglucomutase (PGM) E.C. 2.7.5.1

0.2 M Tris-HCl, pH 8.0	25 ml
Glucose-1-phosphate, disodium salt	250 mg
Glucose-1-6- diphosphate solutiom	0.5 ml
Glucose-6-phosphate dehydrogenase	25 units
1 % $MgCl_2$	0.5 ml
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

Incubate in the dark at 37 ° C until dark blue bands appear

Shikimic acid dehydrogenase (SKDH) E.C.1.1.1. 25

0.2 M Tris-HCl, pH 8.0	25 ml
Shikimic acid	50 mg
1 % $MgCl_2$	0.5 ml
NADP	0.5 ml
MTT	0.5 ml
PMS	0.5 ml

Incubate in the dark at 37 ° C until dark blue bands appear

Staining chemicals kept as solutions

Reagents	Concentration		Storage
NAD (Nicotinamide adenine dinucleotide)	10	mg/ml	4°C
NADP (Nicotinamide adenine dinucleotide phosphate)	10	mg/ml	4°C
NBT (Nitro blue tetrazolium)	10	mg/ml	Dark at 4° C
MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)	10	mg/ml	Dark at 4° C
PMS (Phenazine methosulphate)	5	mg/ml	Dark at 4° C
$MgCl_2$	10	mg/ml	Room temperature
$MgCl_2$	100	mg/ml	Room temperature
glucose-1,6-diphoaphate	0.1	mg/ml	4°C
malic acid,pH 7	0.5	M	Room temperature
cis aconitic acid, pH 8	5%	(w/v)	4°C
isocitrate dehydrogenase	20	unit/ml	4°C

Staining buffer recipes

1. 0.5 M malic acid
   - Dissolve 67.2 g of DL-malic acid in 800 ml distilled water
   - Adjust pH to 7.0 with 10 N NaOH
   - Bring volume to 1 liter

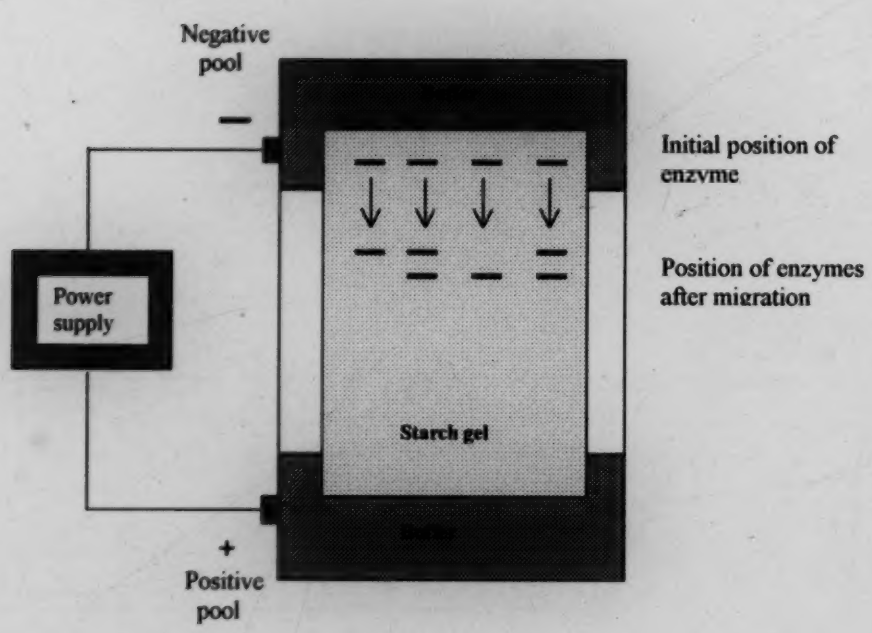
2. 0.2 M Tris-HCl, pH 8.0
   - Trizma base 24.22 g
   - Water 750 ml
   - Adjust pH with HCl
   - Bring volume to 1 liter

3 0.2 M phosphate buffer, pH 7.0 or 6.4
   - Na2HPO4 28.4 g (sodium phosphate, dibasic, anhydrous) Distilled water to 1 liter
   - adjust pH with NaH2PO4 (138 g per liter of sodium phosphate, monobasic, monohydrate)

4. Tris-Maleic , pH 5.3
   - 0.2 M maleic anhydride : 0.2 M Tris-HCl pH 8.0 (1:1 v/v)
   - adjust pH with 1 N NaOH

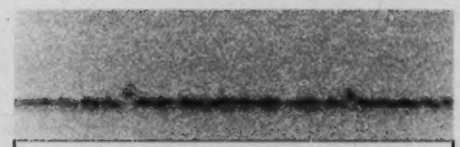
Appendix 2

A starch gel electrophoresis apparatus. The buffers are used to conduct electricity and ensure a given pH.

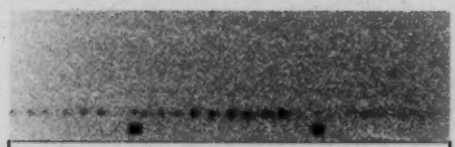


Appendix 3

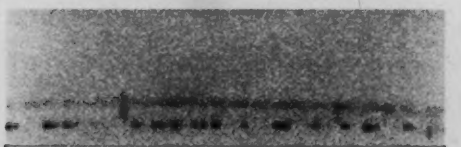
Allozyme zymograms of megagametophyte and embryo tissues of *Pinus merkusii* that were run side-by-side, and the hybrid corn standard.



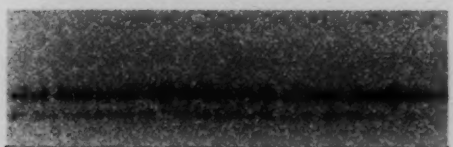
Monomorphic locus of megagametophyte and embryo in ACO enzyme.



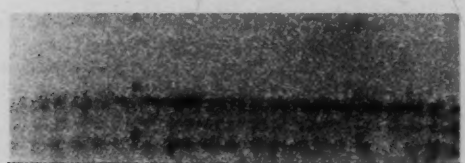
ADH with only one locus that resolved consistently



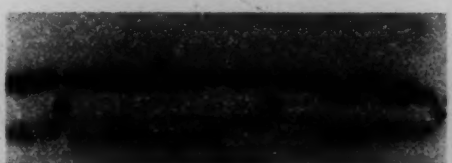
ME with two loci. The second locus is inconsistent.



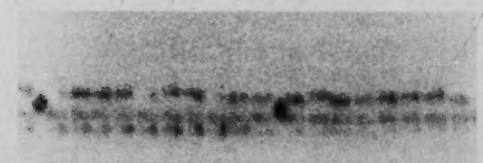
A dimeric enzyme '6-PGD', with two zones of activities.



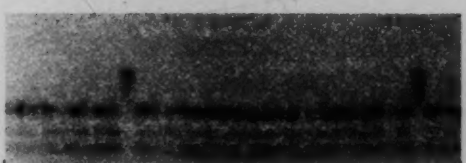
Three zones of activities of AK enzyme



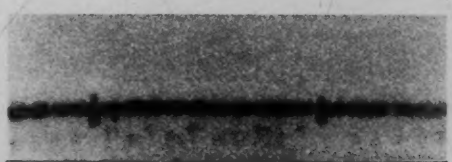
PGM with three loci. The first locus and the third locus are polymorphic, and the second locus is invariant.



SKDH with three loci. The first locus is polymorphic, the others are monomorphic. The embryo tissues stained much stronger than megagametophytes at locus 3.



A dimeric enzyme 'MDH' with three loci. Only the second locus is polymorphic. The third locus is double banded

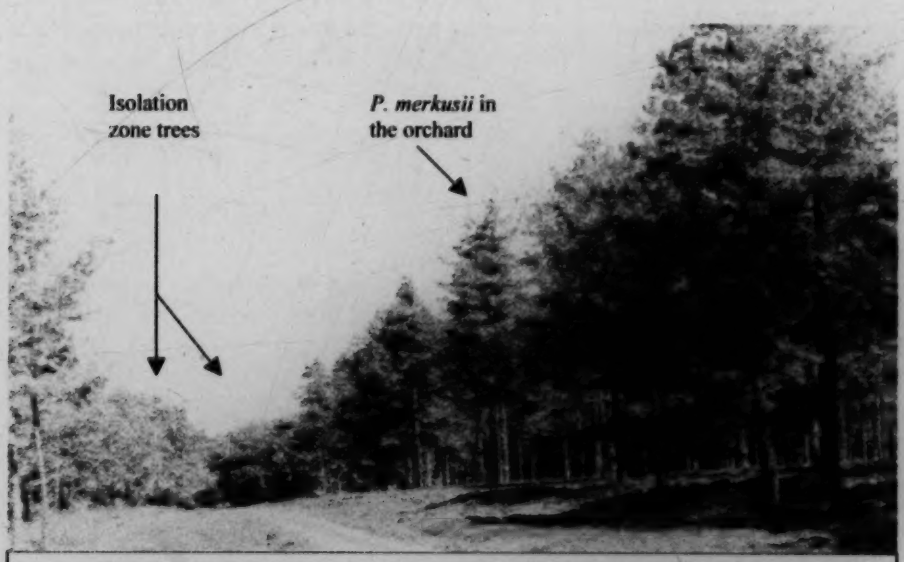


Two zones of activities of 'IDH' with polymorphic locus at *Idh-2*.

Appendix 4

Phenotypes of selected *Pinus merkusii* in Sempolan seedling seed orchard, Jember Forest District, East Java Indonesia

Phenotypes of *Pinus merkusii* in the seed orchard



Phenotypes of *Pinus merkusii* trees and isolation zone trees

Appendix 5

Phenotypes of *P. merkusii* in the local stands surrounding the orchard, at Sempolan, Jember Forest District, East Java, Indonesia

P. merkusii plantation at the north side of the orchard

One of the trees sampled in the south side of the orchard